Name of student, degree, year

Thomas Grover Culton, Doctor of Philosophy, 1947.

Title of thesis

The shank pigment suppressing effect in growing chickens of certain protein supplements.

Thesis and abstract approved: George M. Briggs

George M. Briggs
In charge of thesis

Professor of Poultry Nutrition
Department of Poultry Husbandry

Date: Oct. 8, 1947

THE SHANK PIGMENT SUPPRESSING EFFECT IN GROWING CHICKENS OF CERTAIN PROTEIN SUPPLEMENTS

By

Thomas Grover Culton

Thesis submitted to the Faculty of the Graduate School of the University of Maryland in partial fulfillment of the requirements for the degree of Doctor of Philosophy

1947

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789 East Eisenhower Parkway
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Ann Arbor, MI 48106 - 1346

ACKNOWLEDGEMENTS

It is with sincere appreciation that the author expresses his thanks to Dr. H. R. Bird, who suggested this study and under whose supervision much of the work was conducted; to Dr. George M. Briggs for his advice on the design of the latter experiments and his assistance in the preparation of this manuscript; to Drs. M. A. Jull and T. C. Byerly for their assistance and advice and to Professor J. R. Smyth for making available the facilities of the Maine Agricultural Experiment Station which were used to conduct the last four experiments.

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INTRODUCTION

The color of the flesh and skin of the chicken and yolk of the egg is, and has been for many years, of considerable economic importance to the poultry industry. The following quotation from Palmer (35) illustrates the economic importance of these factors:

> The pigmentation of the yolk of the egg and of the flesh (body fat) is a subject of much practical importance in the egg and poultry industry of this country. The consumer demands highly colored yolks in fancy eggs throughout the year, and the eggs with pale colored yolks, so frequently found on the market during the winter months, are the object of much complaint, particularly in cities. Similarly, in some sections of the country the poultry trade demands a highly colored flesh. For the fancy trade, however, the demand is for a flesh with the least color possible.

The continued economic importance of pigment in the yolk of the egg and the change in market demands between 1915 and 1933 is illustrated by the following statement from Henderson and Willcke (21):

> Interest in the factors which influence the color of the yolk of hens' eggs is being revived. There are at least two reasons for revived interest in this problem. One is the illogical discrimination in certain markets against rich yellow yolks, and the other is, the close association between vitamin A and yellow pigment-producing feeds.

According to Benjamin and Pierce (4) the skin color in market chickens varies from white through cream to golden yellow and to a dark brownish yellow. These authors stated that the white, cream, or golden yellow colors are preferred by discriminating consumers and that the preference is for smooth yellow shanks. That the poultry producer experiences

considerable difficulty in producing the market preferred type of chicken is evidenced by the following statement from Hammond and Harshaw (16).

> It is common knowledge among producers that there is a marked preference for yellow shanks and skin in broilers on the New York live poultry market.
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> . . . . . .
>
> The research reported in this paper was undertaken because the [United States] Department of Agriculture had received many requests for information about means of controlling the color of the shanks and skins of the yellow-skin breeds. Most of these inquiries were for information on how to increase the pigmentation of the shanks and skin.

During the course of investigations at the University of Maryland, it was observed that chicks receiving an experimental diet composed mainly of dextrinized starch and dried skimmilk supplemented with three percent of dehydrated grass, had shanks of a much deeper yellow color than did chicks of similar breeding fed a practical broiler mash containing the same amount of dehydrated grass. In search of a way to measure the difference it was decided to use the yolk-color-rotor designed by Heiman and Carver (18). The chicks receiving the experimental diet had an average score of 14.1 while the chicks receiving the practical diet had an average score of only 7.2. This fact seemed highly irregular in view of the fact that the latter diet contained a high proportion of yellow corn whereas in the former diet the only source of pigment was dehydrated grass.

It has been generally assumed that pigmentation of shanks and body fat is proportional to the amount of pigment in the feed. In the above mentioned comparison, this as-

sumption was obviously untenable and the observed facts led to the hypothesis that some component, or combination of components, in rations composed of common feedstuffs were inhibiting the deposition of yellow pigment in the shanks of young birds. The experiments reported here were designed to test this hypothesis.

REVIEW OF THE LITERATURE

Classification and terminology of the carotenoid pigments. Bogert (7) defines a carotenoid as a nitrogen free polyene pigment, consisting wholly or chiefly of a long acyclic chain of carbon atoms united in an uninterrupted sequence of conjugated double bonds, which system of conjugations function as the chromophore. The chemical reactions, the sources and the chemical and physical methods of investigation of the carotenoids have been reviewed by Palmer (36) and more recently by Strain (56) and Peterson, Hughes and Payne (41). Palmer (37) presented a discussion of the chemical and biological nomenclature for the carotenoids. It is not within the scope of this thesis to fully discuss these phases of the carotenoids and for more detailed information the reader is referred to the above mentioned papers.

According to Peterson, Hughes and Payne (41):

> The carotenoids may be divided into two classes, according to their composition: the hydrocarbons (carotene, lycopene, etc., $C_{40}H_{56}$) which are readily dissolved by ether or petroleum ether but are quite insoluble in aqueous alcohol, and the far larger class of oxygen-containing pigments, the carotenols (xanthophylls), which usually contain at least two hydroxyl groups. Like carotene, practically all carotenoid pigments have 40 carbon atoms.

The following nomenclature of xanthophylls, as used by strain (56),:

> The following system of nomenclature has proved to be the most useful as a guide during the investigation of the leaf xanthophylls reported in this publication. Hydrocarbons which are essentially aliphatic and which owe their color to a conjugated system of double bonds are regarded as carotenes regardless of the number of carbon atoms which they contain. Hydroxy, oxo, and hydroxy-oxo derivatives of the carotenes are named xanthophylls. Carboxy derivatives of carotenes are carotenoid acids. All these pigments and esters of xanthophylls and of carotenoid acids are carotenoids.

seemed to be the most satisfactory for use in this thesis and has been used throughout with the exception of references to the literature, in which case the exact terms of the author have been used.

Strain (56) tabulated 13 carotenes, 30 xanthophylls, 3 xanthophyll esters and 7 carotenoid acids that had been isolated from plants. Of the carotenes, three, alpha-carotene, beta-carotene and gamma carotene and one xanthophyll, cryptoxanthin, were listed as vitamin A-active by Peterson, Hughes and Payne (41).

That the xanthophylls comprise the major portion of the natural yellow pigment of the tissues of the chicken has been amply demonstrated. Of historical interest is the report of Thudicum (58) on the discovery of luteine which he found in the yolks of the eggs. Schunck (52) reported the xanthophyll of egg yolk to be identical with that of leaves. Palmer (35) first demonstrated the physiological relation of the yellow pigments of the tissue of the chicken to that of the carotene and xanthophyll of plants.

The work reported herein is undoubtedly concerned with the xanthophylls, although fractionations of the pigment of the shank skin were not made and have not been reported in the literature. The only report of the chemical nature of the yellow pigment of the shank is that of Palmer and Kempster (39) who stated that:

> The pigment in the epidermis [referring to histological sections of the foot skin] was identified as carotinoid by its ease of oxidation and decolorization with FeCl and hydrogen peroxide.

Carotenoid pigments of the tissues of the chicken. Mattikow (32) presented a complete and comprehensive review of the literature on the coloring matter of egg yolk. Many of the references cited by this author are presented in this review, not of necessity as they were completely and thoroughly covered, but as of historical interest and to present in chronological sequence the more important findings on this subject.

Thudicum (58) first crystallized carotenoid pigments and reported their presence in the yolk of eggs. He assigned the name luteine to these pigments and showed that they were soluble in alcohol, ether and chloroform. Schunk (52) reported that the pigment of egg yolk was identical with that of leaf xanthophyll. Willstatter and Escher (61) were successful in obtaining a crystalline pigment from egg yolk, which, in most of its properties, coincided with leaf xanthophyll but they also suggested the presence of carotene. Palmer (35) confirmed the findings of Willstatter and Escher (62) and also reported that the natural pigments character-

izing the egg yolk, body fat and blood serum of the hen were identical with the carotene and xanthophyll pigments of plants, with the latter class of pigments present in by far the greater proportion. Kuhn and Brockmann (27) confirmed the presence of carotene in the egg yolk.

Kuhn, Winterstein and Lederer (31) reported that the xanthophyll of egg yolk was not a single pigment but consisted for the most part of lutein and another pigment zeaxanthin. Gillam and Heilborn (15) reported the first chromatographic studies of the petroleum-phasic carotenoids of egg yolk and established the fact that the carotene fraction was composed of more than one pigment, and that, cryptoxanthin, a petroleum soluble pigment of yellow corn, Kuhn and Grundmann (28; 29), constituted the primary pigment of the petroleum-phasic carotenoids. The most recent and comprehensive work on the chromatographic separation and identification of carotenoids from egg yolk was reported by Strain (56). This author isolated lutein, zeaxanthin and cryptoxanthin from the yolks of eggs from hens fed a diet composed of meat, corn and small quantities of barley seedlings. When the diet consisted of wheat, bran and milk with 20 percent of fresh alfalfa, or an equivalent quantity of freshly dried alfalfa, the egg yolk contained minute quantities of neoxanthin and flavoxanthin-like compounds and considerable quantities of isolutein, zeaxanthin and cryptoxanthin. Even though these hens received large quantities of carotene only traces were found in the egg. The carotene was composed principally of beta-carotene with traces of alpha-

carotene.

Peterson, Hughes and Payne (41) studied the petroleum-phasic carotenoids of egg yolk and the influence of various components of the diet upon the pigments of this fraction. When the sole source of carotenoids in the diet was yellow corn, the absorption maxima were at 4450 and 4750 Angstrom units and the minimum at 4675 Angstrom units. When green feed supplied the carotenoids, the maxima were at 4500 and 4750 and the minimum at 4600. Chromatographic studies, using the Strain (56) column, were conducted and when green feed was the source of carotenoids about 80 percent of the pigment passed through the column readily, while 20 percent was more strongly adsorbed. When corn was the sole source of carotenoids 55 percent was washed through readily while the middle zone, 20 percent, and the top zone, 25 percent, had to be removed by elution with alcohol. The main fraction in all cases had absorption maxima identical with the original solution and when mixed with carotene and poured through a column could not be distinguished from it, i.e., beta-carotene and the egg pigment came down as a single band. In the case of eggs obtained when corn alone was the only source of carotenoid pigment, these authors assumed that the main fraction was identical with modified beta-carotene of corn; the middle fraction, cryptoxanthin similarly modified, and the top fraction oxidized carotenes. In the case of the eggs obtained on green feeds, as chromatographic studies of the green feed had shown the petroleum-phasic fractions to

consist of pure beta-carotene, the absorption curve of the main fraction was not so easily explained. They suggested that either the slightly acid digestive processes of the hen are responsible for the conversion of the ingested beta-carotene, or perhaps the hen has a more selective mechanism for the separation of these pigments than has been possible to devise in the laboratory. That is to say, since the fowl at best deposits only a very small fraction of the petroleum-phasic carotenoids ingested, it is possible that most green feeds do contain a small amount of this modified beta-carotene, which, although it cannot be separated from beta-carotene by the usual absorption techniques, can be selectively utilized by the fowl. As evidence they cited their inability to introduce beta-carotene into the egg by feeding.

The literature contains very little information on the quantitative relation of these pigments in egg yolk. Peterson, Hughes and Payne (41) stated that the xanthophyll content of egg yolk was approximately ten times the carotene content. Kuhn and Smakula (30) reported the absorption curve of the pigment of egg yolk to be identical with that of a mixture of 32 percent zeaxanthin and 68 percent lutein. Gillam and Heilbron (15) reported that yolks of eggs produced by hens on a heavy corn diet contained two milligrams of carotenoids per 100 grams of which 0.19 milligrams was cryptoxanthin and 0.015 milligrams carotene. When the diet contained large quantities of grass, the total carotenoids increased to 4.2 milligrams per 100 grams of yolk of which 0.14 milligrams was cryptoxanthin and 0.02 milligrams was

carotene.

The carotenoid pigments of the eye have received some attention, due mostly to the fact that certain characteristic changes in the pigments of these tissues are a symptom of the fowl-leucosis complex. Wald and Zussman (59) crystallized three carotenoids, possessing the properties of astacene, xanthophyll and an unidentified hydrocarbon from the cones of the chicken retina and concluded that the astacene and possibly the hydrocarbon were synthesized by the chicken. Neither of these two compounds were identified in egg yolk, liver or blood serum of the chicken. Hollander and Owen (22), basing their conclusions solely on solubility tests concluded that the yellow pigment of the chicken iris was carotenoid in nature and that at least two pigments were present, probably xanthophyll and carotene.

In contrast to the efforts to isolate the various pigments of the egg yolk, no references to attempted fractionization of the carotenoid pigments of the shank skin of the chicken appear in the literature. Palmer and Kempster (39) present the only chemical evidence that the pigment of the shank is carotenoid in nature. That the carotenoid pigment of the shank covering is analagous to that of the yolk is assumed and the following arguments are offered in support of this assumption:

1. Palmer and Kempster (38) reported that they reared two generations of chickens on carotenoid-free diets with every evidence of being normal except for the absence of the

natural yellow pigmentation of the skin.

2. Later Palmer and Kempster (40) reported that carotene in the form of very highly colored butter, from the colostrum milk of a Jersey cow, was without effect in coloring the visible skin parts. The substitution of yellow corn for white corn, in a ration that had been used to deplete White Leghorn cockerels of all yellow pigment in the visible skin parts, resulted in immediate deposition of yellow color in those parts. They reported that the beak, eye rings, ear lobes and shanks of one bird were distinctly yellow, when compared with control birds, after only 72 hours on this diet. In two other birds, xanthophyll became quite noticeable in the beak and shanks after 5 or 6 days. One bird was continued on this dietary regime for 42 days, at which time all visible skin parts were reported as deep yellow and the feathers as exhibiting a smooth, creamy appearance.

3. The fact that the pigmentation of the shanks fade when the dietary source of the pigment in these tissues is removed by excretion through the egg yolk in hens in production, as first reported by Palmer and Kempster (39), is cited as further evidence that the pigments of the shank skin and the egg yolk are identical.

Influence of the carotenoid pigment content of the diet upon the carotenoid pigments of the tissues. Palmer (35) was the first to demonstrate that the pigments of the tissues of the chicken were dependent upon dietary sources of these pigments. In carefully controlled feeding experiments, he

demonstrated the physiological relationship of the natural yellow pigments of the egg yolk, body fat and blood serum of the hen to the xanthophyll of plants and showed that the natural yellow pigments of these tissues were identical with the carotene and xanthophyll of plants, with the latter class of pigments in by far the greater proportion. In comparison to the yolk color of eggs during a preliminary period, reduction of the xanthophyll content of the diet resulted in reduction of the yolk color, while an increase in the xanthophyll content of the diet resulted in an increase in yolk color. Supplementation of the low pigment diet with carotene, from raw carrots, was without effect on egg yolk color. The total yellow pigment content of the blood serum on the various diets was similar to that of the yolk color. The diets were without influence on the color of the body fat, shanks and beaks, which the author attributed to the failure of the diet to cause a deposition of fat in the lower epidermal layers of the shank skin. In the light of our present knowledge on the effect of continued egg production on the pigment of the beak and shanks, the failure of the diet to influence these factors in laying hens is readily explained. Palmer and Kempster (39) observed a similar effect and offered as explanation the theory that is still universally accepted. As for the body fat, references throughout the literature indicate the relative stability of the carotenoids of the adipose tissue, once they are deposited therein.

Palmer and Kempster (39) verified the findings of Palmer

(35) that dietary pigment is without effect in restoring the pigment of the skin and adipose tissues of birds in production and postulated that the failure to do so is due to the deflection of the path of excretion of xanthophyll from the skin to the ovaries.

Palmer and Kempster (40) confirmed the report of Palmer (35) that carotene from butter fat was without effect on the adipose tissue and had little, if any, effect on the visible skin parts. In contrast, xanthophyll from yellow corn, had an immediate effect on the color of both the adipose tissue and visible skin parts. As a result of bioassay, based on changes in yolk color in the eggs produced by hens reared on carotenoid-free rations and laying eggs with xanthophyll-free yolks, yellow corn and green feed were found to be rich in xanthophyll. A small amount of pigment was found in hempseed, barley, gluten feed and red corn but wheat, wheat bran, oats, cottonseed meal, rape seed, meat scrap and blood meal contained only negligible quantities.

Bisbey, Appleby, Wise and Cover (5); Bisbey, Wise and Kempster (6) and Bjollema and Donath (54) reported the quantitative relationship between the xanthophyll content of the diet and the yolks of eggs produced. Bisbey, Appleby, Wise and Cover (5) observed a distinct gradation of color in the egg yolks from hens on different diets. The color of the yolks on a given diet was very uniform. The color of the composite samples of egg yolks from the four rations tested were compared with solutions of varying concentrations of

potassium dichromate. Their dark egg yolks corresponded to the color of 0.1N, medium to 0.07N, light-1 to 0.05N and light-2 to 0.04N potassium dichromate. The dark yolks contained 0.0099 milligrams of carotene and 0.0340 milligrams of xanthophyll per gram and the medium colored yolks 0.0067 milligrams of carotene and 0.0251 milligrams of xanthophyll per gram. The light-1 and light-2 colored yolks contained 0.0044 and 0.0045 milligrams of carotene and 0.0147 and 0.0142 milligrams of xanthophyll per gram, respectively. The dark, medium, light-1 and light-2 yolks were produced on diets containing 65.0 percent of yellow corn and 10 percent of alfalfa leaf meal, 65.0 percent of yellow corn, 35.0 percent of yellow corn and 25.0 percent of yellow corn, respectively, as sources of pigment. Bisbey, Wise and Kempster (6) reported that a definite relationship existed between the depth of the color of the yolks of the eggs produced and the ration of the hens.

Sjollema and Donath (54) confirmed the previous report of Palmer (35) and Bisbey, Appleby, Wise and Cover (5) that there was a greater proportion of xanthophyll than carotene in egg yolk. On the pigment-free ration the carotene content of the egg yolk decreased to as low as three micrograms per yolk and the xanthophyll content to a level of 20 to 40 micrograms. On a diet containing 25 percent of yellow corn and 5 percent of alfalfa the carotene content increased to approximately 35 micrograms and the xanthophyll content to approximately 400 micrograms per yolk. When the diet con-

tained 25 percent of yellow corn as the only source of pigment, the carotene content of the yolks produced was approximately 20 micrograms and the xanthophyll content approximately 300 micrograms per yolk. When five percent of alfalfa was the sole source of pigment in the diet, the yolks produced contained approximately 20 micrograms of carotene and 250 micrograms of xanthophyll each.

Hughes and Payne (23) and Peterson, Hughes and Payne (41) reported the results of extensive studies on the quantitative relationship of the amount of carotenoid pigments ingested and the amount of these pigments excreted in the egg yolk. When hens that had been depleted of carotenoid pigments to a point where they were laying eggs of a uniform light color, were fed 10, 20, and 30 grams of yellow corn for a period of 21 days, in addition to a carotenoid-free basal diet, 12.0, 6.4, and 4.8 percent, respectively, of the carotene and cryptoxanthin consumed was recovered in the yolks of eggs produced during the feeding period and 21 days thereafter. At this time the yolks were as low in pigment as those of eggs produced by negative control birds. The percentage recovery of the xanthophylls was much greater than that of the petroleum-phasic pigments, being 17.4, 21.4, and 23.5 percent for the 10, 20 and 30 gram intake levels, respectively. With increased consumption of the latter class of pigments the percentage recovery increased while with increased consumption of the carotenoids, the percentage recovery decreased. In determining the effectiveness of

carotenoid pigments from different sources, the technique was altered and determinations were made on the yolks of eggs produced during a period of a week and compared to the carotenoids consumed during that period. Prior to the test week the carotenoid-depleted birds were fed the experimental ration for a period of three weeks. When green barley was the source of pigment, 2.62 percent of the petroleum-phasic fraction and 15.84 percent of the alcohol-phasic pigments were recovered in the yolk. When dehydrated alfalfa was the pigment source, the recoveries were almost identical to those reported for barley, 2.50 and 15.75 percent for the petroleum-soluble and alcohol-soluble pigments, respectively. In contrast, 7.07 percent of the pigments of the petroleum fraction and 25.73 percent of the alcohol fraction of yellow corn was recovered in the yolks. Each source of pigment was fed in such amounts as to furnish approximately equal quantities of the carotenoid pigments. When leaf xanthophyll, zeaxanthin, cryptoxanthin and beta-carotene were administered orally at the rate of one milligram per day for 7 days, 11.45 percent of the leaf xanthophyll, 24.35 percent of the zeaxanthin and none of the cryptoxanthin and beta-carotene were recovered in the yolk of the eggs produced during the feeding period and two weeks thereafter. Approximately twice as much of the zeaxanthin as of the leaf xanthophyll was recovered in the yolk, thus accounting for the observed differences in the utilization of the alcohol-phasic pigments of corn as compared to those of green barley and dehydrated

alfalfa.

Brockman and Volker (10) had also demonstrated that crystalline lutein and zeaxanthin could be introduced into the yolk but were unable to increase the carotene, lycopine and violoxanthin content of egg yolk by feeding of the purified compound.

Gillam and Heilbron (15) reported the results of a chromatographic study of the vitamin-A active pigments of the egg yolk and also studied the effect of the different components of the diet on these pigments. The chromatogram of the pigment of the yolk usually showed a broad yellow-brown zone near the top, a strong yellowish ring below and a weak pink-brown ring lower still. Each ring was separately eluted with methyl alcohol and spectroscopically examined and were found to exhibit absorption maxima identical with each other and with those of beta-carotene. On a diet containing large quantities of yellow corn, the chromatogram was similar in that it exhibited the three bands. The author concluded that the first band was xanthophyll plus oxidation products, the second cryptoxanthin and the lower one beta-carotene.

Palmer and Kempster (38, 39) indicated that no carotenoid pigment other than that in the feed was deposited in the yolk. These authors noted that some pigment continued to be deposited in the yolk from hens on a diet of extremely low carotenoid content and concluded that this residual pigment was not carotenoid in nature and that the quantity was

not reduced by continued feeding of carotenoid-free diets. Riddle (43, 44); Romanoff (45) and Warren and Conrad (60) showed that the carotenoid-bearing yellow yolk is deposited during a period of seven to ten days just prior to ovulation. If the conclusions of Palmer and Kempster (38, 39) were correct then hens reared on diets of high and low carotenoid content would produce eggs of identical carotenoid content after eleven or more days of low carotenoid feeding. That the carotenoid content of the diet is not the sole source of carotenoid pigment for the egg yolk was demonstrated by Bohren, Thompson and Carrick (8). When hens reared on high and low carotenoid diets were placed on a low carotenoid diet, the carotenoid content of the yolk of the former group of hens decreased rapidly during the first ten days of low carotenoid feeding. The carotenoid content of the yolk of the eggs produced by hens reared on the high carotenoid diet averaged 4.2 gamma per yolk greater than that of yolks from hens reared on a low-carotenoid diet, from the 10th day until termination of the experiment, 84 days after the start of the feeding of the carotenoid-low diet. These results were highly significant as indicated by a t value of 5.55, with the value required for significance being only 2.8 at the one percent level. These authors offered two possible sources of pigment that might account for this difference, namely, the deposition of the pigment from the growing diet in the immature ova or the pigment in the body tissues, presumably liver and metabolizable fat. The previously cited

work of Riddle (43, 44); Romanoff (45) and Warren and Conrad (60) heavily discount the former theory. Too, there is no information in the literature that would lend credence to the latter. Bohren, Thompson and Carrick (8) presented no experimental evidence supporting the latter theory, although, they did consider it the most plausible of the two explanations offered.

Rubin and Bird (46), feeding a practical diet, with and without alfalfa leaf meal, studied the yellow-pigment stores in the liver of young chickens. The former ration contained 133.0 units of yellow pigment per gram and the latter 19.5 units of yellow color per gram on a carotene color basis. The liver stores of yellow pigment of chickens on both diets increased the first week and then decreased rapidly until the chicks were approximately three weeks of age. At three weeks of age this trend was reversed and thereafter the total yellow pigments of the liver increased rapidly. The original level was surpassed during the sixth week and at seven weeks of age, both groups had accumulated equal stores of yellow pigment in their livers. The yellow pigment liver-stores of birds on the alfalfa-supplemented diet did not reach as low a level as those of the birds on the unsupplemented diet. The shanks of the former group were more deeply pigmented than those of the latter, according to these authors, despite the lack of difference in the liver stores.

Bolin, Lampman and Berg (9) confirmed the report of Rubin and Bird (46) that the total yellow pigments in the

liver decreased progressively until the 19th day of age, regardless of the amount of pigment in the diet. In lots of chickens from hens receiving 200, 500, and 2,250 micrograms of carotene daily from alfalfa meal, placed on diets containing 0, 25, 100 and 2,250 micrograms of carotene per 100 grams of diet, from the same source, the total yellow pigments in the liver decreased progressively until the 19th day. After 19 days, there was a gradual increase in liver pigment stores in the three groups receiving the higher levels of carotene and by 56 days the three lots receiving 2,250 micrograms of carotene per 100 grams of diet contained appreciably the same quantity, despite the marked differences at the start of the experiment. The chickens on the lower levels of carotene intake did not store any appreciable quantities of yellow pigment in their livers, irrespective of the dam's carotene intake, although the quantity did increase from the 19th to the 56th day. The failure of the birds on the lower level to increase their liver stores of yellow pigment might be considered in disagreement with the aforementioned work of Rubin and Bird (46). The differences in the carotenoid pigments of the different diets, however, invalidates any comparison between the two experiments on this basis. The pigment-low diet of Rubin and Bird (46) contained 19.5 units of yellow color per gram, mostly from yellow corn. The basal diet of Bolin et. al. (9) was a "white" ration. When the latter diet was supplemented with sufficient alfalfa meal to supply 100 micrograms of carotene per 100 grams of

diet, approximately 200 micrograms of xanthophylls, mostly leaf xanthophylls or lutein, per 100 grams of diet would be added, according to Peterson et. al. (41). The alfalfa leaf meal used by Rubin and Bird (46) analyzed 51.5 micrograms of carotene and 133.0 units of total yellow color per gram. If the xanthophyll content of alfalfa leaf meal is generally twice that of the carotene content as proposed by Peterson et. al. (41) then a yellow color unit of Rubin and Bird (46) closely approximates 1 microgram of carotenoids on the basis of their reported analysis. Applying this information to the pigment-low diet of these authors gives a value of 1950 microgram of carotenoids per 100 grams of diet. Since the majority of this pigment is from yellow corn, again referring to Peterson et. al. (41) for pertinent information, relatively twice as much of the alcohol-phasic fraction of yellow corn as that of the alcohol-phasic fraction of alfalfa is deposited in the egg yolk. It has not been so demonstrated, but if one assumes a situation analogous to that observed in egg yolk in the relative effect of these products on the liver stores of yellow pigment then the difference in the "effective" pigment content of the pigment-low diet of Rubin and Bird (46) would be much greater than the actual carotenoid pigment when compared to the pigment-low diets of Bolin et. al. (9). On this basis, the fact that the pigment content of the livers of the chicks of Bolin et. al. (9) on their pigment-low diet did not show comparable increases to those of Rubin and Bird (46) is easily explained. To

explain the fact that the liver stores of yellow pigment on the pigment-low and alfalfa supplemented diets of Rubin and Bird (46) attained the same level at seven weeks of age, one would have to assume that a threshold of liver stores of yellow pigment existed and that the pigment-low diet contained sufficient carotenoids to reach this threshold in liver stores of yellow pigment.

Rubin and Bird (46) suggested two possible explanations for the observed decrease of the liver stores of yellow pigment during the first three weeks of the chicken's life. These authors suggested that this decrease could be explained by the removal of the pigment accompanying the normal decrease in fat content of the liver during this period of life, but if this were the true situation, these authors could not account for the failure of vitamin A to do likewise. Their other suggestion was that the carotene is transferred from the yolk to the liver and is not converted to vitamin A until after the first week. According to their suggestion, the carotene is then gradually converted to vitamin A, thus accounting for the decrease in total yellow pigment stores of the liver. A test of this theory, based on the information available in the literature, renders it untenable.

Peterson, Hughes and Payne (41) demonstrated that it was impossible to introduce beta-carotene into the egg yolk by feeding same but did increase the petroleum-phasic carotenoids of egg yolk by feeding yellow corn, grass silage, alfalfa meal and fresh grass. The maximum level attained was 0.14

milligrams of petroleum-phasic carotenoids per 100 grams of yolk when alfalfa meal was the source. Assuming 18 grams of yolk per egg as reported by Sjollema and Donath (54), conversion of this data to petroleum-phasic carotenoids per yolk for comparison to the data of Sjollema and Donath (54) and Almquist, MacKinney and Necchi (1) yields a value of approximately 25 micrograms of petroleum-phasic carotenoids per yolk. Sjollema and Donath (54) reported that the carotene content of egg yolk from hens on a pigment-low ration to be less than 15 micrograms per yolk and that this value could be increased to a maximum of 35 micrograms per yolk by inclusion of 7.5 percent of alfalfa meal and substitution of yellow corn for white corn in their basal diet. Almquist, MacKinney and Necchi (1) confirmed the report of Peterson et. al. (41) that the carotene content of egg yolk could not be increased by the feeding of crystalline carotene but their results are in conflict with those of Peterson et. al. (41) and Sjollema and Donath (54) as far as the maximum carotene content of egg yolk is concerned. Detailed results on this point were not presented by Almquist et. al. (1) but they stated that the carotene content of the yolk in no case exceeded 6 micrograms per yolk. One each of their experimental groups had received 760 I. U. of vitamin A per bird per day in the form of carotene from alfalfa meal or dried carrots by oral administration.

It is impossible to reconcile the findings of the latter authors with those of Peterson et. al. (41) and Sjollema and

Donath (54) on the basis of carotene intake or analytical methods. Assuming an average consumption of 0.25 pounds of feed per hen per day, the diet of Peterson et. al. (41) furnished approximately 740 I. U. of vitamin A per day; that of Sjollema and Donath (54) a minimum of 750 micrograms of carotene or approximately 1250 I. U. of vitamin A as carotene per day, whereas the diet of Almquist et. al. (1) supplied 750 I. U. of vitamin A daily from the same source by oral administration. On the basis of carotene intake, the yolks of the eggs from hens on the diet used by Almquist et. al. (1) should have equaled those of Peterson et. al. (41) in carotene content. The analytical procedure of each of these authors was very similar and the reported carotene content refers to the petroleum-phasic carotenoids in both cases.

If one assumes that the yolks of the eggs from which the chickens used by Rubin and Bird (46) were hatched contained 35 grams of carotenoid pigment per yolk, the maximum value reported in the literature, and that this carotene was transferred quantitatively from the egg yolk to the liver during the first week of life and then gradually converted to vitamin A, complete decolorizing of the egg yolk pigment could not account for the total decrease in liver stores of yellow pigment that these authors reported. Their data shows a decrease from a maximum of approximately 110 yellow units at one week of age to a minimum of 20 yellow units in the group fed the highly pigmented ration and of 10 yellow units in the group fed the lesser pigmented ration.

The data of Bolin et. al. (9) show similar minimums and maximums for their groups from hens receiving 500 micrograms of carotene daily from alfalfa. The values of the latter authors are expressed as micrograms of beta-carotene and it has been shown herein that the yellow unit of Rubin and Bird (46) approximates one microgram of carotenoid pigment. This decrease of liver stores of yellow pigment, totalling approximately 100 micrograms of carotenoid pigment, could not be accounted for on the basis of conversion to vitamin A of carotene withdrawn from the yolk and stored in the liver during the first week of life, as the yolk does not contain that quantity of yellow pigment capable of conversion to vitamin A. Too, the majority of these liver pigments are xanthophylls and are not capable of conversion to vitamin A, as has been amply demonstrated by research work previously reported herein. Further evidence in support of the fallacy of this explanation was also offered by Bolin et. al. (9) in the form of definite proof that the chicken does convert carotene to vitamin A during the first week of its life. The liver stores of vitamin A in chickens hatched from the eggs of hens receiving 200 micrograms of carotene daily decreased in inverse proportion to the amount of carotene in their diet when the diet contained 0, 25 or 100 micrograms of carotene per 100 grams, however, when the chickens received a diet containing 2,250 micrograms of carotene per 100 grams, the liver stores of vitamin A trebled between the first and eighth day of life. This increase was apparent as

early as the third day and continued at a rapid rate until the termination of the experiment at 56 days.

After a portion of the work reported herein had been completed, Sherwood and Fraps (53) reported that certain lots of meat scraps destroyed carotene in chicken diets. In their experiments, one out of five lots of meat scrap tested, proved unsatisfactory. When incorporated into a diet containing 175 micrograms of carotene in oil per 100 grams, at a level of nine percent the chicks on the diet containing the unsatisfactory meat scraps grew very slowly and the mortality was high, with most of the chickens exhibiting symptoms of vitamin A deficiency. Mortality was not excessive in the chicks fed the other lots of meat scraps. The results were similar when the diet contained five percent of alfalfa leaf meal containing 160 micrograms of crude carotene per 100 grams and no carotene in oil. There was no correlation between the free fatty acid content and the carotene destroying power of feeds. It was also observed that the carotene destroying power of a given feed vary from time to time.

Heiman and Tighe (19, 20) concluded that when other factors which might affect shank pigmentation were held constant, there was an essentially linear relationship between the amount of yellow pigment carriers in the feed and the concentration of carotenoid pigment in the shank. Also, the carotenoid pigment concentration in the shank skin was shown to increase with age, when the concentration of carotenoid

pigment in the diet was held constant.

Hammond, Miller and Whitson (17) reported that large quantities of cod liver oil and small quantities of sulphur in the diet of the hen interfered with the transfer of pigment from her diet through her eggs to the shanks of her chickens. This experiment was poorly designed in that the amount of pigment in the diet of the various groups was not held constant and his conclusions are open to criticism as far as the effect of cod liver oil is concerned. The experiment using flowers of sulphur was much better controlled than the experiment using cod liver oil and his conclusions concerning flowers of sulphur are warranted by the data presented.

Maw and Nikolaiczuk (34) were able to reduce the carotenoid content of the shank skin by intramuscular injection of one-half cubic centimeters of 15 percent ethyl alcohol or an equal amount of 0.1 percent trieresol when compared to controls that had been intramuscularly injected with one-half cubic centimeters of distilled water. The ethyl alcohol injections also reduced the carotene content of liver and blood serum. This study was conducted as the result of some unexpected results obtained from pituitary extracts that had been preserved in 15 percent ethyl alcohol. No explanation of the mechanisms involved was suggested by these authors and a search of the literature failed to reveal any information that might account for this phenomena. To determine the relation of the effect of the alcohol and trieresol to

the previously discussed work of Hammond and Harshaw (16) or the experimental results reported herein will necessitate further experimentation.

Biological significance of the carotenols in chickens. In view of the relatively wide distribution of these compounds in chickens and their selective utilization when compared to the carotenes, it is surprising that it has been impossible to attach any physiological significance to their presence.

The use of the fading of these pigments from the visible skin parts as a result of continued production, as an aid in selecting laying hens is adequately discussed by Rice, Hall and Marble (42). It is very surprising that one can trace so completely the history of the egg production of the hen by observation of the amount of yellow pigment in these tissues and yet be unable to demonstrate any physiological significance of these pigments, in this or any other body function.

The only evidence to the contrary was presented by Ball (2). This author graded White Leghorn pullets that were ready to lay into five grades based on the degree of shank pigmentation and concluded as follows:

> That shank color is related to mortality was indicated by the greater proportion of birds with pale shanks in the Susceptible strain than in the Resistant strain. Moreover within both of these strains, the pullets which were classified as having pale shanks died at a greater rate than did the pullets which were well pigmented. No relation was found to exist between shank color and the incidence of colds or egg production to 500

days of age.

No discussion of the phenomena was offered nor was evidence presented as to whether the observed mortality was the cause or the effect.

The possibility that some agent was causing the mortality and also suppressing the pigmentation of the shanks cannot be ignored. Too, there were genetic differences in the resistance to disease of the two strains of birds studied by Ball (2), and although the genetics of shank color was not investigated, the fact that there were differences in shank color between the Resistant and Susceptible strains suggests a possible linkage between the color of the shanks and resistance to disease.

Only one of the many carotenols has been demonstrated to possess vitamin A activity. The vitamin A potency of cryptoxanthin was first demonstrated by Kuhn and Grundmann (29), who also pointed out that the vitamin A potency of yellow corn was due mainly to cryptoxanthin rather than to beta-carotene. Since yellow corn constitutes a large percentage of many practical poultry diets, much of the total requirement of the poultry industry for vitamin A is supplied by this compound.

Palmer and Kempster (38) were the first to demonstrate that carotenoid pigments were not necessary for growth, fecundity and reproduction in domestic fowls. After two unsuccessful attempts to rear chickens to maturity on a pigment-low ration, a third was marked by success due to the

inclusion of pork liver in the diet. With this innovation these authors were able to rear two generations of birds on their pigment-low diet with no evidence of abnormality other than the absence of the natural yellow pigmentation of the skin. The mature hens of the first generation showed normal fecundity and no abnormalities with respect to the fertility of the carotenoid-free eggs developed.

Russell and Weber (50); Kline, Schultze and Hart (26); and Karrer, Schopp and Morf (25) demonstrated that xanthophyll possessed no vitamin A activity for chickens.

Schumacher, Scott, Hughes and Peterson (51), using crystalline luteol and zeaxanthol, confirmed these reports that xanthophyll possessed no vitamin A potency for chickens and demonstrated that carotenols are not essential nutrients in the diet of the male for normal reproduction.

Histology of the yellow pigmentation in the shanks of chickens. Barrows (3) made a histological study of the shank color in the domestic fowl and reported that the yellow shanks resulted from the presence of "zooxanthin" in both layers of the epidermis, or in the corneum alone, diffused through all parts of the cells and intercellular substances. In brightly colored shanks both epidermal layers exhibited a rich supply of the oil. In young birds the amount in the Malphigian layer was large but as the chickens grew older this disappeared and old laying hens carried a very small quantity in the horny layer, the rete appearing white to the naked eye. Old hens which have never laid, possessed a deep

orange color in both parts of the epidermis, indicating that the original pigment had not been used up and that an additional supply had been deposited.

Palmer and Kempster (39) in general, confirmed the findings of Barrows (3) but interpreted the xanthophyll to be present in granular form rather than in an oil or fat solution. In support of this view they reported Nile blue to be dichromatic with respect to fat and xanthophyll, the fat staining red and the xanthophyll deep blue. Nile blue stained sections of highly pigmented shank skin showed xanthophyll as dark blue granular masses in the outer part of the epidermis and in the cells of the rete of Malphigi and also lining the blood capillaries in the lower corium layer. In these sections fat droplets in the subcutaneous layer stood out prominently as scarlet colored droplets. These authors also reported some sections that showed a reddish tinge in the epidermis which indicated to them the presence of some fat. In explanation of these sections they offered the suggestion that xanthophyll was probably serving as the solvent and the small amount of fat was present as the solute while in deeply pigmented adipose tissue, which stained scarlet with Nile blue, the xanthophyll was dissolved in fat. This observation immediately suggests that perhaps Barrows (3) and Palmer and Kempster (39) are both correct and that the reported differences might be due to differences in fat depots of the birds at the time of preparation of the sections. Too, differences in histological

technique might account for this discrepency. Barrows used tissue slices that had been fixed and preserved in 10 percent formalin and mounted in glycerol. He used no stain. Although Palmer and Kempster (38) did not describe their histological technique, it did differ to the extent that the latter authors based their conclusions on studies of stained sections.

Palmer and Kempster (38) also reported histological studies on the fading of the xanthophyll from the shanks of highly pigmented White Leghorn cockerels when placed on their carotenoid-free diet. The pigment from the corium disappeared first followed by that in the outer layer of the epidermis and gradually extending into the rete of Malphigi. The last pigment to disappear being the xanthophyll at the base of the Malphigian layer. They interpreted these observations as meaning that when the pigment supply is cut off the pigment present in the corium layer is normally deposited in the rete of Malphigi. At the same time the pigment in the outer layer of the epidermis either wears off by normal replacement of the outer cells from those lower down, or is oxidized because of closer contact with the air, or possibly both. The xanthophyll in the rete of Malphigi then gradually becomes a part of the outer layer of the epidermis and is lost by the above process, or processes, until the skin becomes free from visible yellow pigment. These authors expressed the opinion that the now universally recognized disappearance of xanthophyll from the ear lobes, beak, and

shanks of laying hens, as a result of continued production, is analogous to that observed in their cockerels and is a result of the removal of the supply of pigment for these parts. These authors postulated that for non-laying hens the skin is the normal path of excretion of xanthophyll but that production deflects excretion from these tissues to the egg yolk.

The observation of Russell, Taylor, Walker and Polskin (49) that carotene is excreted through neither the kidney nor the intestine indicates that the skin and egg yolk are the only paths of excretion of the carotenoids.

Influence of dietary factors, other than the amount of carotenoid pigment in the diet, upon deposition of these pigments in the various tissues of the chicken. The first evidence that dietary factors other than the amount of the carotenoids in the diet influenced the deposition of yellow pigment in the various parts of the body was published after these experiments were underway, by Hammond and Harshaw (16). These authors demonstrated the quantitative relationship between the quantity of pigment-bearing feeds in the diet and the color of the shank and skin in the growing chicken, providing the amount of pigment-suppressing dietary factors was held constant. Fortified cod liver oil was the most potent source of the pigment-suppressing factor, with ordinary cod liver oil about one-half and sardine oil one-third as potent as the fortified cod liver oil. Activated animal pro-vitamin D or irradiated ergosterol did not affect

the shank and skin color even at levels supplying several times the number of units of vitamin D required. The fortified cod liver oil was freed of a large portion of the pigment suppressing factor by heating at 230°C in a vacuum for three hours.

Deuel, Halliday, Hallman, Johnston and Miller (11) demonstrated that the feeding of vitamin A in large doses in the form of shark liver oil to Guernsey cows was followed by a pronounced lowering in the carotene level of the milk. Later Deuel, Hallmann, Johnston and Mattson (12) concluded that the depression in milk carotene was a resultant of feeding vitamin A itself rather than of other components in the shark liver oil, inasmuch as it also occurred after the administration of a vitamin A concentrate. Moreover, it was noted that a similar response could be elicited in Holstein cows even though the normal carotene level of their milk was approximately 50 percent less than that of the Guernsey. They also presented evidence that the intensity of the effect was proportional to the dosage through a certain range. Since a similar variation was manifested in blood carotene these authors suggested that the observed phenomena were best explained on the basis of the development of a new enzyme system capable of destroying carotene. They postulated that the formation of the enzyme system was stimulated by the high concentration of vitamin A in the tissues. As evidence, they cited the slow rate of the development of the maximum decrease, observed in four to six weeks after the

initiation of vitamin A feeding, and the tardy return to normal levels after cessation of such supplementation, a period of approximately eight to ten weeks. The possibility of increased activity of an enzyme system already present as a result of the high vitamin A intake was also considered.

Mattson and Deuel (33) extended these studies on carotenoid metabolism to chickens. Two-week old chicks, that had been reared on a commercial starting diet, were fed a pigment-low diet for two weeks and then put on a diet in which 25 percent of alfalfa meal had been substituted for 25 percent of wheat flour in the original diet. One-tenth cubic centimeter of shark liver oil containing 9300 I. U. of vitamin A was administered orally to the experimental birds. The results are in agreement with those previously observed (Deuel et. al. (11, 12) in cattle and with those of Hammond and Harshaw (16), if one interprets their results in the light of later knowledge, and Rubin and Bird (47) if it is assumed that factors which affect the pigment content of the liver and blood would likewise affect the pigment content of the skin. The effects observed by Mattson and Deuel (33), as a result of feeding shark liver oil to chickens, were of greater magnitude and more absolute than in cattle. After receiving the pigment-low diet for two weeks followed by the pigmented diet for two weeks, the carotenol content of the liver and blood plasma of the control chickens was 19.5 * 2.8 micrograms per gram and 790 micrograms per 100 cubic centimeters, respectively. In the

experimental groups these values were reduced to 4.35 ± 1.1 micrograms per gram and 0.0 micrograms per 100 c.c. for liver and blood plasma, respectively. The carotene content of the liver of chickens on this dietary regime was unchanged, both values being a minimum, as compared to the groups fed the pigment-low diet for two weeks. The carotene content of the blood plasma decreased from 11.7 micrograms per 100 cubic centimeters in the controls to 0.0 micrograms per 100 cubic centimeters in the experimental groups. Absorption studies indicated that the differences were not the result of an upset of absorption in the vitamin A supplemented animals. Over a period of one week, approximately 75 percent of the carotene and 40 percent of the carotenols consumed in both control and experimental groups, were absorbed or destroyed in the digestive tract.

Deuel, Hrubetz, Mattson, Morehouse and Richardson (13) reported a similar depression in carotenoid content of the eggs, liver blood and body fat of hens as a result of feeding rations containing high levels of vitamin A. Shark liver oil containing 20,000 I. U. of vitamin A per gram was added to a diet containing approximately 10 milligrams of carotenoids per pound in such quantities as to increase the potency of the diet of the respective groups to 1000, 2000, 15,000, 30,000 and 100,000 I. U. of vitamin A per pound. After 75 days the 2,000 and 15,000 I. U. level groups were discontinued and one-half of each group combined to form two new groups, which were fed diets containing 60,000 and 200,000 I. U. of vitamin A per pound. There was no alteration in

the pigment content of egg yolks from the groups fed 1000 and 2000 I. U. per pound but a marked decrease was observed in the 15,000 I. U. level group and the decrease became progressively greater with each succeeding group. The yolks of the eggs from hens receiving the diet containing 200,000 I. U. of vitamin A per pound contained only 25 percent as much carotenoid pigment as those from the controls. The carotenoid content of the blood serum and liver was progressively lowered in comparison with the controls in the groups receiving 30,000 I. U. and higher levels but the results were statistically significant only with the two groups receiving the highest levels, because of the small number of observations. The decrease in blood serum and liver carotenoids was greater in the group which had received 200,000 I. U. per pound for three months than in the group that had received 100,000 I. U. per pound for six months. However, only the group receiving the 100,000 I. U. level showed a marked lowering of the carotenoid content of the body fat.

Rubin and Bird (47) showed that the pigment-suppressing factor in fish-liver oils is vitamin A. Using basal diet C of Hammond and Harshaw (16), these authors obtained equivalent depression of the color of the shank from 9000 I. U. of vitamin A per 100 grams of feed whether the vitamin A was from vitamin A concentrate, a vitamin A and D feeding oil or crystalline vitamin A alcohol. They also demonstrated that the observed differences were manifested in part, at least, in the blood or other tissues, and was not exclusively an

intestinal phenomena. Two groups of chickens were fed a non-pigmented diet for six weeks, one group receiving only enough vitamin A to support normal growth and the other 100,000 U. S. P. units of vitamin A per 100 grams of diet. At the end of this period both groups were fed the diet C of Hammond and Harshaw (16) and shank pigment measured every two days for 36 days. At the end of this period the low vitamin A intake group showed 1.5 micrograms of xanthophyll and 1,112 blue units of vitamin A per gram of liver and the high vitamin A intake group showed 1.4 micrograms of xanthophyll and 5.406 blue units of vitamin A. The chicks with the lower vitamin A stores began to accumulate yellow pigment in their shanks after two days on the pigmented diet and reached a maximum at 16 days. The chickens with the higher vitamin A stores accumulated no measurable quantities of yellow pigment in their shanks until 12 or 14 days and had not attained the level of the former group at the end of the experiment.

Since Rubin and Bird (47) demonstrated that crystalline vitamin A inhibited the deposition of yellow pigment in the shanks of growing chickens, it is logical to assume that the effects reported by Deuel et. al. (11, 12 and 13), Hammond and Harshaw (16), Hammond, Whitson and Miller (17) and Mattson and Deuel (33) on the carotenoid content of their experimental materials was due to vitamin A. All of these authors were experimenting with vitamin A concentrates and the observed effects were in relation to vitamin A intake.

TABLE 1. Composition of basal diet 3 and modifications thereof used in Experiment 1.

Component	Group 1	2	3	4	5	6
	Basal diet 3	3	3	3	3	3
	%	%	%	%	%	%
Dextrinized corn starch*	41	39	42	47	45	41
Dried skimmilk	54	54	36	31	33	37
Calcium carbonate	1	1	1	1	1	1
Sodium chloride	1	1	1	1	1	1
Cod liver oil	**	2	2	2	2	2
Dehydrated grass	3	3	3	3	3	3
Soybean oil meal #1	-	-	15	-	-	-
Menhaden meal #1	-	-	-	15	-	-
Meat scrap #1	-	-	-	-	15	-
Wheat flour middlings #1	-	-	-	-	-	15

*Two percent of dextrinized corn starch was replaced by two percent of dried brewer's yeast as a source of B-complex vitamins on 1/30/40.

**Cod liver oil administered orally 1/4 c.c. the first week, 1/2 c.c. the second week and 1 c.c. per week thereafter.

MATERIAL AND METHODS

Experimental trials. Ten feeding experiments have been conducted, six at the University of Maryland, College Park, Maryland, and four at the Maine Agricultural Experiment Station, Orono, Maine. In all experiments the chicks were distributed at random by a system that resulted in approximately equal distribution of starting live weights and of the sexes in each lot. The chicks were brooded in electrically heated battery brooders in a steam heated room. The diets were supplied ad libitum. In the experiments conducted at the University of Maryland, the chicks were weighed weekly. In those at the Maine Agricultural Experiment Station weighings were made biweekly, with the exception of experiment 9 in which they were weighed weekly.

Experiment 1. This experiment was designed to experimentally test the previously postulated hypothesis that dietary factors, other than the amount of pigment in the diet affected the amount of pigment deposited in the shanks of growing chicks and to isolate the components of the ration responsible for this suppression of pigmentation. This experiment was started on 1/10/40 and terminated 2/22/40. It consisted of six groups of 25 White Wyandotte male x New Hampshire female crossbred chicks. Color scores were taken when the chickens were five and six weeks of age.

The diets used are described in Table 1. All supplements were included in the diet at the expense of dextrinized corn starch and dried skimmilk in such a manner as to

TABLE 2. Compositions of basal diets 7 and 8 and modifications thereof used in experiment 2.

Component	Group 1	2	3	4	5	6
	Basal diet 7	7	7	7	8	8
	%	%	%	%	%	%
Ground white corn	52	62	65	54	-	-
Ground yellow corn	-	-	-	-	53	63
Dehydrated grass	3	3	3	3	-	-
Dried skimmilk	42	18	15	24	44	20
Meat scraps #1	-	15	-	-	-	15
Menhaden meal #1	-	-	15	-	-	-
Soybean oil meal #1	-	-	-	15	-	-
Ground oyster shell	1	-	-	1	1	-
Steamed bone meal	-	-	-	1	-	-
Salt	1	1	1	1	1	1
Manganese sulphate	0.012	0.012	0.012	0.012	0.012	0.012
Cod liver oil	1	1	1	1	1	1

maintain the protein level constant in all groups. The diets were mixed in such quantities as to last approximately one week.

Experiment 2. This experiment was designed to test the possibility of simplifying the preparation of the basal diet. In some groups ground white corn was substituted for the dextrinized starch and in others ground yellow corn was substituted for the dextrinized starch and dehydrated grass. These substitutions made the basal diet much less laborious to prepare and decreased the laxative effect of the skimmilk. It also served as a check on different sources of pigment.

The experiment was started on 2/23/40 and consisted of six groups of ten Rhode Island Red chicks per group. Groups one to four, inclusive, were terminated on 4/11/40 and groups five and six were continued to nine weeks of age, being terminated on 5/9/40. Color scores of the shanks of the chickens of all groups were taken at five and seven weeks of age and of groups five and six only at eight and nine weeks of age.

The diets used are described in Table 2. All supplements were included in the diet at the expense of the corn and dried skimmilk in such a manner as to maintain the protein level constant in all groups. The diets were mixed in such quantities as to last approximately one week.

Experiment 3. Since Experiments 1 and 2 showed conclusively that certain feedstuffs had suppressed the deposition of yellow pigment in the shanks of young chicks fed the ex-

[redacted]

TABLE 3. Diets used in experiment 3.

Component	Group 1	2	3	4	5	6	7	8
	Diet A	B	C	D	E	F	G	H
	%	%	%	%	%	%	%	%
Ground yellow corn	26.5	26.5	37.5	40	36.5	35.5	28.5	31.5
Ground heavy oats	10	10	10	10	10	10	10	10
Wheat bran	10	10	10	10	10	10	10	10
Wheat flour middlings	10	10	10	10	10	10	10	10
Alfalfa leaf meal	5	5	5	5	5	5	5	5
Corn gluten meal	5	5	5	5	5	5	5	5
Soybean oil meal #1	15	15	—	—	—	—	15	15
Meat scrap #1	—	—	19	—	7.5	—	—	—
Menhaden meal #1	—	—	—	17	6	10	2	5
Dried skimmilk	13	—	—	—	7.5	13	10	5
Dried buttermilk	—	13	—	—	—	—	—	—
Cod liver oil	1	1	1	1	1	1	1	1
Ground oyster shell	2	2	—	—	1	0.5	2	2
Steamed bone meal	2	2	—	—	—	—	1	—
Curbay B-6.	—	—	2	2	—	—	—	—
Manganese sulphate	.012	.012	.012	.012	.012	.012	.012	.012
Salt	0.5	—	0.5	—	0.5	—	0.5	0.5

[redacted]

perimental diets, Experiment 3 was designed to determine if similar effects could be observed in broilers fed standard broiler mashes.

Experiment 3 was started on 7/2/40 and terminated when the chickens were ten weeks old. It consisted of eight groups of 25 Rhode Island Red chicks per group. Color scores of the shanks were taken at four, five, nine and ten weeks of age.

The composition of the diets of the respective groups are given in Table 3.

Experiment 4. Only one brand each of meat scraps, menhaden meal and soybean oil meal had been used in Experiments 1, 2 and 3. In Experiment 4, these brands were retested and also included were samples of each of 50 percent protein meat scraps and of steam dried menhaden meal. Also, a combination of meat scrap and soybean oil meal was fed one group to test the supplementary effect of these two substances.

This experiment was started on 9/24/40 and continued for a period of six weeks thereafter. It consisted of eight groups of 25 New Hampshire chicks per group. Color scores were taken at four and six weeks of age.

The composition of the diets are given in Table 4.

Experiment 5. Experiment 5 was designed to test the pigment inhibiting effect of the ether soluble fraction of meat scraps. This experiment was started on 3/18/41 and continued for four weeks. It consisted of seven groups of

TABLE 4. Composition of diets used in Experiment 4.

Component	Group 1	2	3	4	5	6	7
	Basal diet 8	8	8	8	8	8	8
	%	%	%	%	%	%	%
Ground yellow corn	53	64	64	61	61	57	61
Dried skimmilk	44	19	19	22	22	24	22
Meat scrap	-	-	-	15*	15**	-	7.5*
Menhaden meal	-	15	15	-	-	-	-
Soybean oil meal No. 1	-	-	-	-	-	15	7.5
Ground oyster shell	1	-	-	-	-	1	-
Steamed bone meal	-	-	-	-	-	1	-
Salt	1	1	1	1	1	1	1
Cod liver oil	1	1	1	1	1	1	1
Manganese sulphate	0.012	0.012	0.012	0.012	0.012	0.012	0.012

*Meat scrap (50% protein) No. 1.

**Meat scrap (50% protein) No. 2.

Menhaden meal No. 1.

Menhaden meal No. 2.

[redacted]

TABLE 5. Composition of diet 8A and modification of same used in experiment 5.

Component	Group 1	2	3	4	5	6	7
	Diet 8A	8A	8A	8A	8A	8A	8A
	%	%	%	%	%	%	%
Ground yellow corn	47.75	47.75	47.75	47.75	47.75	47.75	47.75
Dried skimmilk	45.0	24.0	45.0	24.0	24.0	45.0	45.0
Calcium carbonate	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Sodium chloride	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Cod liver oil	0.25	0.25	0.25	0.25	1.0	0.25	0.25
Manganese sulphate	0.012	0.012	0.012	0.012	0.012	0.012	0.012
Corn starch	5.0	11.0	0.5	13.2	11.0	1.6	2.5
Meat scrap #1	-	15.0	-	-	15.0	-	-
Ether extract of meat scrap*	-	-	4.5	-	-	-	-
Residue of ether extraction*	-	-	-	12.8	-	-	-
Non-saponifiable fraction*	-	-	-	-	-	3.4	-
Saponifiable fraction*	-	-	-	-	-	-	2.5

* These fractions supplemented at a level equivalent to 30 percent of meat scrap. The yield of the various fractions was as follows:

1 gram residue equivalent to 1.17 gram meat scrap.
1 gram ether extract equivalent to 6.74 gram meat scrap.
1 gram saponified fraction equivalent to 12 gram meat scrap.
1 gram imsaponified fraction equivalent to 9 gram meat scrap.

[redacted]

25 White Leghorn cockerels per group. Color scores were taken at four weeks of age.

The fractions used in this experiment were prepared from a lot of meat scrap No. 1. The meat scrap was extracted with ether in a soxhlet type extractor for 48 hours. One-half of the ether extract was saponified by refluxing with 10 percent alcoholic potassium hydroxide. The unsaponifiable fraction was extracted with ether and the saponifiable matter acidified and fed as free fatty acids. The yields were as follows:

One gram of residue equivalent to 1.17 grams meat scraps.

One gram of ether extract equivalent to 6.74 grams of meat scraps.

One gram of saponified fraction equivalent to 12.0 grams of meat scraps.

One gram of unsaponified fraction equivalent to 9.0 grams of meat scraps.

The basal ration was modified by the substitution of 5.0 percent corn starch for 5.0 percent yellow corn in order that the source of yellow pigment in the diet could be held constant. All extracts were substituted for corn starch at a level equivalent to 30 percent of meat scraps, with the exception of the residue of ether extraction. The residue was substituted at a level equivalent to 15 percent of meat scraps due to its high protein content. The skimmilk and starch content was varied in each diet in such a manner as to maintain the protein at a constant level. The diets of the various groups are described in detail in Table 5.

TABLE 6. Composition of diets used in experiment 6.

Component	Group 1	2	3	4	5	6
	Diet I	J	K	L	M	N
	%	%	%	%	%	%
Ground yellow corn	40	40	40	40	40	40
Ground wheat	20	20	20	20	20	20
Wheat bran	5	5	5	5	5	5
Alfalfa leaf meal	3	3	3	3	3	3
Dried skimmilk	10	10	10	10	10	10
Casein	9	1.5	1.5	-	9	9
Corn starch	7.5	3.5	3.5	1.5	6.5	7.5
Ground oyster shell	1	-	-	1	-	1
Steamed bone meal	2.5	-	-	2.5	-	2.5
Sodium chloride	1	1	1	1	1	1
Cod liver oil	1	1	1	1	1	1
Meat scrap #1	-	15	-	-	-	-
Meat scrap #3	-	-	15	-	-	-
Blood meal #1	-	-	-	15	-	-
Ash meat scrap #1	-	-	-	-	4.5	-
Ferric ammonium sulfate	-	-	-	-	-	23g/cwt.

Experiment 6. Experiment 6 was started on 10/15/41 and consisted of 25 White Wyandotte male x New Hampshire female chicks per group. Color scores were taken at four, five and six weeks of age.

Included were groups receiving a third sample of meat scraps, dried blood meal, the ash of meat scraps No. 1, and ferric ammonium sulphate with iron equivalent to the iron content of the ash of meat scraps No. 1. Meat scraps No. 1 contained 30 percent ash and 0.04 percent iron.

The basal ration used in Experiment 6 was modified in such a manner as to more closely approach a practical ration than did ration 8 or 8A. Casein was substituted for a portion of the dried skimmilk, and wheat and alfalfa meal substituted for a portion of the yellow corn. The diets of the respective groups are described in Table 6.

Experiment 7. Experiment 7 was designed as a preliminary experiment to reacquaint the writer with the experimental technique and to establish supplies of feedstuffs that manifested the pigment-suppressing effect.

This experiment was started on 9/18/46 and consisted of seven groups of 16 Rhode Island Red chickens per group. It was of 5 weeks duration and color scores were taken only at the termination of the experiment. The method of determining color scores will be discussed in detail later.

The incorporation of casein into the basal diet in Experiment 6 had proved beneficial in eliminating the undesirable laxative qualities of the excess skimmilk and it

TABLE 7. Composition of diets used in Experiment 7.

Component	Group						
	1	2	3	4	5	6	7
	%	%	%	%	%	%	%
Ground yellow corn	50	50	50	50	50	50	50
Corn gluten meal	13	13	13	13	13	13	13
Alfalfa meal	5	5	5	5	5	5	5
Dried skimmilk	27	8	6	2	24	8	2
Fish meal base vitamin carrier	-	-	15	-	-	-	-
Meat and bone scrap No. 1	-	15	-	-	-	15	-
Fish meal No. 3	-	-	-	15	-	-	15
Cod liver oil (85D)	-	-	-	-	3	3	3
Corn starch	-	8	9	13	-	5	10
Ground limestone	3	1	2	2	3	1	2
Steamed bone meal	2	-	-	-	2	-	-
Manganese sulphate (g.)	6	6	6	6	6	6	6
Delsterol (g.)	23	23	23	23	23	23	23

was deemed desirable to retain this feature of the diet. At the beginning of Experiment 7, however, it was impossible to secure casein. Soybean oil meal was eliminated as a source of protein because of its previously demonstrated (Experiment 2) pigment-suppressing properties. In view of this it was decided to use yellow corn gluten meal as a source of protein. This feedstuff had not been previously tested to determine what effect it might have on pigmentation, but since yellow corn had been satisfactorily used, it was assumed that corn gluten meal would be satisfactory. As casein was available for Experiment 8 and subsequent experiments, no more corn gluten meal was used and comparative tests to determine the effects of the inclusion of this feedstuff in the diet were not conducted.

One sample of 45 percent protein meat and bone scraps, one sample fish meal, one sample of a fish meal base vitamin carrier, one sample of cod liver oil and the supplementary effects of each of these products and cod liver oil were tested.

All supplements were incorporated into the basal diet at the expense of dried skimmilk and corn starch, in such a manner as to maintain the protein at a nearly constant level. The diets used in this experiment are described in Table 7.

Experiment 8. This experiment was designed to test the effect of heat treatment on the supplements which had been shown in Experiment 7 to possess the pigment suppressing properties. Experiment 8 was started 7/13/47 and terminated

[redacted]

TABLE 8. Basal diet and supplements thereto used in Experiment 8.

Component	Groups						
	1 & 3	2 & 5	4 & 7	6 & 14	8 & 9	10 & 13	11 & 12
	%	%	%	%	%	%	%
Ground yellow corn	62.925	62.925	62.925	62.925	62.925	62.95	62.925
Alfalfa leaf meal	2.000	2.000	2.000	2.000	2.000	2.00	2.000
Dried skimmilk	12.000	8.000	12.000	12.000	8.000	20.00	8.000
Casein	2.000	-	2.000	2.000	-	10.00	-
Fish meal No. 3	-	-	-	-	-	-	20.000
Meat and bone scraps No. 1	-	-	-	20.000	-	-	-
Autoclaved fish meal No. 3	-	21.700	-	-	-	-	-
Dry-heated fish meal No. 3	-	-	-	-	18.880	-	-
Autoclaved meat and bone scraps No. 1	20.00	-	-	-	-	-	-
Dry-heated meat and bone scraps No. 1	-	-	19.120	-	-	-	-
Corn starch	-	3.800	0.880	-	6.620	-	5.500
Steamed bone meal	-	-	-	-	-	2.50	-
Mico	1.000	1.500	1.000	1.000	1.500	2.50	1.500
Delsterol	0.050	0.050	0.050	0.050	0.050	0.05	0.500
Manganese sulphate	0.025	0.025	0.025	0.025	0.025	-	0.025

[redacted]

five weeks later. It consisted of 14 groups of eight Rhode Island Red cockerels and seven diets were tested in duplicate. Color scores were taken at five weeks of age.

Two methods of heat treatment of the meat and bone scraps and fish meal were used. One was autoclaving and the other was dry-heat treatment.

Prior to treatment, a quantity of the sample calculated as sufficient to complete the planned experiment, 13 kilograms in this case, was thoroughly mixed and sampled according to the following plan. After thoroughly mixing, the sample was divided into six approximately equal parts by passing lines of cleavage through the center of the pile at approximately 60 degrees angle. The opposite sixths were then combined and mixed thoroughly. From these three portions of the original sample, 4000 grams of each were used, one for autoclaving, one for dry-heat treatment and the other as control.

The dry-heat treatment consisted of 48 hours at 76°C in a steam-heated drying room. The samples were spread on wrapping paper in uniform layers approximately one-half inch in thickness and placed on drying racks for treatment. The drying racks were so arranged as to allow free circulation. Three racks were required for each sample and the racks holding each sample were placed in tiers in adjacent frames. Temperature was recorded approximately one-half inch above the surface of the middle drying rack. The temperature was very constant and the heat evenly distributed over the area

of the two racks used. These racks were in the approximate center of the drying room. The samples of fish meal and meat and bone scraps were treated simultaneously.

For autoclaving, the samples were spread in uniform layers of approximately one inch in thickness in white porcelain pans and autoclaved at 15 pounds of pressure. The meat and bone scraps were autoclaved for six hours and the fish meal was autoclaved for a period of only four hours due to mechanical failure of the autoclave. After removal from the autoclave, the samples were spread in thin layers and air dried for 48 hours prior to incorporation into the diet. During the drying process, the samples were stirred frequently and efforts were made to press out all lumps that were formed. These efforts were not 100 percent effective, therefore after drying was complete, the samples were pressed through the finest mesh sieve that would pass completely the original product.

Every precaution was taken to make each transfer of the samples quantitative. It is recognized that this is impossible in samples of this size, nevertheless, it was assumed that any change in weight was due to treatment. The treated samples were incorporated into the diet at a level calculated, on the basis of percentage recovery, to be equivalent to a level of 20 percent of the original product. The percentage recovery of the various samples was as follows:

Dry-heat treated fish meal - 94.4 percent.

Dry-heat treated meat and bone scraps - 95.6 percent

Autoclaved fish meal - 108.5 percent.

Autoclaved meat and bone scraps - 101 percent.

The small difference between the autoclaved meat and bone scraps and the original sample was ignored. The dried skim-milk and casein content of the diets was varied to maintain the protein level constant between the groups. Corn starch was used as filler in the diets where necessary. The diets of the respective groups are described in Table 8.

Experiment 9. Experiment 9 was designed to determine if the meat and bone scraps used in Experiments 7 and 8 was capable of destroying carotene as reported by Sherwood and Fraps (53) and, if so, was this carotene-destroying power associated with the pigment-suppressing factor.

This experiment consisted of 18 groups of Rhode Island Red chickens. It was started on 8/20/47 and terminated 9/24/47.

With the exception of the negative control diets, all diets were fed to duplicate groups of nine chickens per group. The negative control diets were fed to only one group of 15 chickens each.

The chickens were observed closely for the appearance of the external symptoms of vitamin A deficiency and the date of their appearance recorded. Autopsies were performed on all chickens that died during the experimental period. In analysis of the results, only those birds that had exhibited ataxia prior to death and had deposits of urates in the kidneys and ureters on autopsy were considered as having died

of vitamin A deficiency. The latest and most complete description of these symptoms is given by Taylor and Russell (57).

The basal diet used was a vitamin A deficient diet which was being used in another project at the Maine Agricultural Experiment Station and supported good, though not maximum, growth when supplemented with adequate vitamin A. Alfalfa leaf meal was used as a source of vitamin A. The alfalfa leaf meal was incorporated into the diet at the expense of casein and corn starch in such a manner as to maintain the protein content of the various diets at a constant level. The composition of the diets of the respective groups is given in Table 9.

At the beginning of this experiment it was planned to measure shank pigmentation at five weeks and each week thereafter until the termination of the experiment. Scoring the shank color of the groups that were expected, on the basis of previous experimentation, to have the lighted and darkest colored shanks at five weeks of age, revealed no measurable differences between these groups, all scores being a minimum. Therefore, the measurement of the shank color score in this experiment was abandoned.

The original plan was to continue Experiment 9 for eight weeks, but since the results, as far as both the carotene destroying power and the pigment-suppressing factor in the meat and bone scrap, were essentially negative, it was discontinued after five weeks.

[redacted]

TABLE 9. Composition of vitamin A deficient diet and supplements thereto used in Experiment 9.

Component		Groups									
		7	1 & 13	8 & 11	2 & 12	6 & 14	5	9 & 17	3 & 15	4 & 18	10 & 16
Ground wheat	%	60	60	60	60	60	60	60	60	60	60
Ground oats	%	10	10	10	10	10	10	10	10	10	10
Dried skimmilk	%	10	10	10	10	10	3	3	3	3	3
Casein	%	10.5	10.38	10.25	10.13	10	2.5	2.38	2.25	2.13	2.0
Meat and bone scraps No. 1	%	--	--	--	--	--	20.0	20.0	20.0	20.0	20.0
Curbay B-Y	%	2	2	2	2	2	2	2	2	2	2
Steamed bone meal	%	3	3	3	3	3	--	--	--	--	--
Mico	%	3	3	3	3	3	1	1	1	1	1
Starch	%	1.5	1.12	0.75	.37	--	1.5	1.12	0.75	.37	--
Alfalfa leaf meal	%	--	0.5	1.0	1.5	2.0	--	0.5	1.0	1.5	2.0
Manganese sulfate	(g)	6	6	6	6	6	6	6	6	6	6
Delsterol	(g)	10	10	10	10	10	10	10	10	10	10

[redacted]

Experiment 10. Experiment 10 included a new sample each of 50 percent protein meat scraps and 60 percent protein fish meal as well as the meat and bone scraps and fish meal used in Experiments 7 and 8 and autoclaved samples of each of these products. The treated samples in this experiment were autoclaved for eight hours at a pressure of 15 pounds. Otherwise the treatment was identical with that of the autoclaved samples of Experiment 8. The recoveries, which ranged from 3997 to 4020 grams as compared to an original sample of 4000 grams, were considered as 100 percent, and were included in the ration on that basis.

This experiment was started on 8/29/47 and terminated 9/26/47. It consisted of nine groups of 17 Rhode Island Red chickens per group. Color scores were taken at the termination of the experiment.

The diets of the respective groups are described in detail in Table 10.

Methods of measuring shank pigmentation. The color of the shanks of the chickens in Experiments 1 through 6, inclusive, was measured by use of the yolk-color-rotor of Heiman and Carver (18). The yolk-color-rotor consists of a series of 24 glass discs, varying in color from white through very deep orange, mounted on a black, wooden disc. The wooden disc is mounted at a slight angle and can be rotated so as to bring every disc into the same position. This allows for each reading to be taken in exactly the same light, provided the source of light remains constant. All

[redacted]

TABLE 10. Composition of diets used in Experiment 10.

Component	Group								
	1	2	3	4	5	6	7	8	9
	%	%	%	%	%	%	%	%	%
Ground yellow corn	61.88	61.88	61.90	61.88	61.88	61.88	61.88	61.88	61.88
Alfalfa leaf meal	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Dried skimmilk	8.00	12.00	20.00	8.00	8.00	8.00	8.00	12.00	8.00
Casein	-	2.00	10.00	2.73	-	-	2.73	2.00	-
Fish meal No. 3	20.00	-	-	-	-	-	-	-	-
Fish meal No. 4	-	-	-	-	20.00	-	-	-	-
Meat and bone scraps No. 1	-	-	-	-	-	-	-	20.00	-
Meat scraps No. 4	-	-	-	-	-	-	20.00	-	-
Autoclaved fish meal No. 3	-	-	-	-	-	20.00	-	-	-
Autoclaved fish meal No. 4	-	-	-	-	-	-	-	-	20.00
Autoclaved meat and bone scraps No. 1	-	20.00	-	-	-	-	-	-	-
Autoclaved meat scraps No. 4	-	-	-	20.00	-	-	-	-	-
Corn starch	5.50	-	-	3.27	5.50	5.50	3.27	-	5.50
Steamed bone meal	-	-	2.50	-	-	-	-	-	-
Mico	1.50	1.00	2.50	1.00	1.50	1.50	1.00	1.00	1.50
Manganese sulfate	0.02	0.02	-	0.02	0.02	0.02	0.02	0.02	0.02
Salt	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Delsterol	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10

[redacted]

readings were taken by the same individual without his having previous knowledge of the diet received. At the last reading the chicks from the various groups were mixed and their color score determined thus eliminating entirely the possibility of the individual readings in a group being biased by the group as a whole.

The numerical scores of the yolk-color-rotor pertinent to this data may be described in terms of yellow color as follows: 5 and 6 practically no yellow color; 7 and 8, pale yellow; 9 and 10, yellow; 11 and 12 deep yellow; 13 and 14 orange yellow; and 15 and 16, orange.

In the experiments conducted at the Maine Agricultural Experiment Station the pigment scores have been determined by means of an arbitrary score for two reasons. For a period of time there was no yolk-color-rotor available due to the fact that the pigments required for construction of this equipment were unavailable. When a yolk-color-rotor was available (purchased from the designers) the tints were such that it was impossible to match the color of the shanks of the strain of birds used with the colors of the discs of the yolk-color-rotor. The inability to match the shank color of the chickens used with the colors of the yolk-color-rotor was indeed surprising. Several different strains and breeds of birds had been used in Experiments 1 through 6 inclusive, and no similar difficulty was encountered. Too, several different rations had been used in Experiments 1 to 6, inclusive, and satisfactory matches were obtained on all diets

tested. It was therefore concluded that the pigments used in the manufacture of this yolk-color-rotor differed from those of the ones used in the manufacture of the yolk-color-rotor used in Experiments 1 to 6, inclusive, and this procedure was abandoned.

Two methods of measuring the intensity of the color of the shank skin were used in the experiments conducted at the Maine Agricultural Experiment Station. In Experiment 7 the shank color was determined by the use of an arbitrary score, ranging from 0 to 3. Shank colors of white and pale yellow, light yellow, dark yellow and orange were represented by the scores of 0, 1, 2 and 3, respectively. Since this experiment was of a preliminary nature and due to the fact that a yolk-color-rotor was to be available for future experiments, very little planning went into the devising of this scheme. Although it was not used again, the data from succeeding experiments indicate that it was satisfactory and did serve its purpose.

The method used for scoring the pigmentation of the shanks of chicks in Experiments 8 and 10 was an arbitrary score similar to that used by Hammond and Harshaw (16) and Hammond, Miller and Whitson (17). These authors used an arbitrary score ranging from 0 to 8, and described the color represented by each number as follows:

No yellow pigment ------- 0

Very slight yellow ------ 1

Slightly yellow --------- 2

Medium yellow ---------- 3

Deep yellow ---------- 4

Very deep yellow -------- 5

Very slightly orange ---- 6

Slightly orange --------- 7

Medium orange ---------- 8

The method used in Experiments 8 and 10 differed from that of Hammond and Harshaw (16) in that a range from 1 to 8 was used and instead of assigning arbitrary colors to each number, and scoring the shanks from a mental picture of this number, a "standard" bird was selected to represent each pigment score in each experiment.

Prior to the reading of the shank pigment score, all chickens in the experiment were mixed in a large battery compartment in order to eliminate the possibility of individual readings being biased by the readings of the group. The chickens were individually removed from this large compartment and placed into one of eight possible compartments depending upon the color of the shanks. The plan was to place birds of nearly equal shank pigmentation in the same compartment, with equal gradation between the compartments, basing the gradation on a mental picture of the lightest and most highly colored shanks in the experiment. After the chickens in the experiment had been divided in this manner, two persons examined each of the groups as a whole for uniformity of shank color and moved to higher or lower groups any that were deemed not within the range of that particular

group. When the greatest uniformity possible was attained by this method, a standard chicken from each group was selected at random. These standard chickens were matched, one against the other, for equal gradation of color. Any standard chicken selected at random that was adjudged unsatisfactory for any reason was put back into the group from which it was taken and another selected. This was repeated until a chicken was obtained that had shanks of such color as was adjudged midway between that of the next lower and next higher standard. When so selected, the eight standard chickens represented eight equally spaced gradations of pigmentation ranging from the minimum to the maximum. All chickens were then matched against the standard of their respective group and in a few cases chickens were moved to a higher or lower group. After this grouping, the scores were recorded as 1, 2, 3, 4, 5, 6, 7 or 8 with increasing amounts of pigmentation represented by the higher score. In no case was it necessary to move any chicken, when checking against the standard, more than one group, either higher or lower.

After using the two methods, it was recognized that both had certain advantages and that both left much to be desired. The data among different experiments was comparable when the yolk-color-rotor was used as a standard, but with some birds, and especially those that had lighter colored shanks, it was difficult to match the color of shank with any color of the yolk rotor. The number of chickens

falling in this difficult range in any one experiment was very small, however. When the arbitrary color score was used, no difficulty was encountered in checking against the standard. This method does have the obvious disadvantage in that it would be influenced to a degree by the spread in color between the lightest and darkest colored shank in any experiment. If a color score of one represented the lightest colored shanks and a color score of eight the deepest colored shanks, then it is obvious that a color score of one would not necessarily represent the same shade in any two experiments. Likewise, no definite shade of yellow could be ascribed to any other color score that would be comparable between different experiments. Also, the range between any two color scores would be influenced among different experiments by the spread between the lightest and darkest colored shanks. In all experiments reported herein in which the shank color score was determined by use of this arbitrary score, the same basal diet and the same strain of birds have been used. Under these conditions it is assumed that the lightest and darkest colored shanks in different experiments would be very similar and likewise the color represented by each color score. As pointed out above, however, this definitely would not apply to experiments conducted using diets with varying amounts of pigment. The results of Experiments 8 and 10, when compared with those of the previous experiments, are very similar, thus indicating that the arbitrary score was entirely satisfactory and that the same

identity was being measured.

During the period in which these experiments were conducted several methods of measuring pigmentation in the shanks have been published. One of these is the method used by Hammond and Harshaw (16), described above, and after which the arbitrary score used in Experiments 8 and 10 was patterned.

Heiman and Tighe (19, 20) outlined a method for measuring the relative amount of carotenoid pigment in the shanks. Their method was based on the extraction of the pigments from a definite area of skin in acetone. The chickens were killed and the skin removed from the shank by cutting through the shank skin around the metatarsus at both the proximal and distal ends, and parallel to the axis on the back side. The skin was then peeled off. All adhering tissue was removed, the skin washed free of blood and other stains and the excess moisture removed with paper toweling. The skin was then spread on a wooden block and a circle of skin, five-eighths inch in diameter, cut with a punch at a point corresponding to the front and center of the shank. The discs of skin were then extracted with 10 c.c. of acetone by allowing to stand in the dark for 48 hours with frequent shaking. The acetone was then decanted and the color intensity read with a photoelectric colorimeter using a light filter No. 42 blue.

This method is highly accurate when the pigment extracts from the right and left legs of birds were compared.

When six sets of four legs, each ranging from colorless to deep yellow and having equally spaced gradations of pigmentation as far as could be determined with the eye, were compared with the concentration of pigment in the extract, the relation was curviliniar. Since the scale readings on the colorimeter are directly proportional to the concentration of the substance being measured, the curve suggested a logarithmic relationship. In this case, each successive quantity of pigment in the shank skin exerted less coloring effect visible to the eye.

Ball (2) made a shank-color scale by taking shanks from recently killed pullets and matching them with plates of a color dictionary. This author used five grades described as follows:

Grade	Interpretation
0	very pale
1	pale
2	light yellow
3	yellow
4	orange

After the standard shanks were selected, they were mounted an inch apart on a black cardboard. When making comparisons, the outer side of the shank was compared with the same region of the shanks on the color-scale. Fresh color-scales were made prior to each reading and were not used over four hours after preparation.

Either the method of Heiman and Tighe (19, 20) or Ball

(2) would probably have been satisfactory for use in the experiments reported herein. However, the original consideration in undertaking these experiments was to determine to what extent dietary factors, other than the amount of pigment in the diet, influenced the visible pigmentation of the shanks. Since the results of Heiman and Tighe (19, 20) indicated that their method did not give the same results as visual observations in more deeply pigmented shanks it was not used. The shank-color grading system of Ball (2) is considered very comparable to the method of Hammond and Harshaw (16), Hammond, Miller and Whitson (17) and the method used in these experiments. The main differences being that Ball (2) used a color standard to select his standard shanks and used only five grades while Hammond and Harshaw (16) and Hammond, Miller and Whitson (17) used nine grades and a visual classification. In comparison, the method used herein employed only eight grades and visually selected live chicks were used as standards. The eight grades then represented the lightest and mostly highly colored shanks and six equally spaced gradations of pigmentation between them. As no difficulty was encountered in dividing the chickens in any experiment into eight groups, a greater degree of accuracy was attained by the use of eight rather than five color grades. Had the spread between the lightest colored and most deeply pigmented shanks been smaller, then the method of Ball (2) could have been applied with equal precision and accuracy.

Statistical analysis of the experimental results. Analysis of variance, Fisher (14) and Snedecor (55), has been applied to all experiments except Experiment 9. The data from Experiment 9 are of such a nature that statistical analysis is unnecessary and unwarranted. Due to its greater simplicity and ease of handling, the F test for significance of Snedecor (55) has been used in preference to the z test of Fisher (14). As pointed out by Snedecor (55) the F test and z test gives the same result.

The least significant difference between means has been calculated by the following formula from Snedecor (55): error of the difference between two means equals the square root of (error variance x $n_1 + n_2 \div n_1 n_2$) when n_1 and n_2 represent the number of chickens in the two groups. The value of t, for degrees of freedom in error variance at the five percent or one percent level, as desired, x standard error of the difference between the two means equals the least significant difference in color score between the two means. When n_1 equals n_2 the formula for computation of the standard error of the mean difference becomes the square root of (error variance x 2 ÷ n) when n equals the number of birds in each group.

RESULTS

Experiment 1. The results of Experiment 1 are summarized in Table 11. The results are considered conclusive

TABLE 11. Summary of results of Experiment 1.

Group	Basal Diet	Modification	Ave. color scores		Least significant differences@				Ave. wt.	Mortality
					5 weeks		6 weeks			
			5 weeks	6 weeks	5%	1%	5%	1%	6 weeks gms.	%
1	3	Cod liver oil orally	10.2**	9.6**	1.17	1.55	1.02	1.34	229	0
2	3	None	8.0	7.1	-	-	-	-	205	14
3	3	15% Soybean oil meal No. 1	8.6	8.2*	1.17	1.55	1.02	1.34	290	0
4	3	15% Menhaden meal No. 1	5.9**	5.8*	1.29	1.71	1.14	1.51	345	32
5	3	15% Meat scraps No. 1	6.0**	5.8*	1.29	1.71	1.12	1.48	250	32
6	3	15% Wheat flour middlings	10.8**	9.0**	1.17	1.55	1.02	1.34	237	0

@Least significant difference between group 2 and the respective groups.

*Difference, when compared to group 2 statistically significant between the 5% and 1% level.

**Difference, when compared to group 2, statistically significant beyond the 1% level.

evidence that dietary factors, other than the amount of pigment contained in the diet, affected the pigmentation of the shanks. In Experiment 1, one sample each of soybean oil meal, menhaden meal, meat scraps and wheat flour middlings were tested for their pigment-suppressing properties. Also tested was the effect of the mixing of cod liver oil in diet in comparison to oral administration.

Of the feedstuffs tested, the menhaden meal and meat scraps were the only ones that were found to possess pigment-suppressing potency. The average shank color score of the groups of chicks receiving these two feedstuffs was significantly lower beyond the one percent level than that of the controls at five weeks of age. However, at six weeks of age this difference had decreased to a point where it was no longer significant beyond the one percent level but was significant between the five percent and one percent levels.

The shank color score of the groups receiving the soybean oil meal and wheat flour middlings was greater than that of the controls. The differences were significant between the five percent and one percent levels at six weeks of age in the groups receiving the soybean oil meal and beyond the one percent level at both five and six weeks in the groups receiving wheat flour middlings.

The shank color score of group 1, which had received cod liver oil by oral administration, was significantly greater beyond the one percent level than that of the control group.

Experiment 2. Since Experiment 1 had definitely indicated that certain components of the diet did suppress pigmentation in the shanks, Experiment 2 was designed to determine whether the preparation of the basal diet might be simplified in order that the study of this phenomena might be expanded. Two basal rations, seven and eight were tested in this experiment. The dextrinized starch of diet three was replaced by white corn in diet seven. In diet eight yellow corn replaced the dextrinized starch and dried grass of diet three.

The results of Experiment 2 are presented in Table 12. That diets seven and eight were satisfactory for measurement of the pigment-suppressing property is demonstrated by these results. In agreement with the results of Experiment 1, the meat scraps again suppressed the pigmentation of the shanks to an extent that was statistically significant beyond the one percent level at five weeks on both diets seven and eight. On diet seven, the difference was still significant beyond the one percent level at seven weeks but was insignificant on diet eight. However, the difference between the diet eight control group and the meat scrap supplemented group on that diet was significant between the five percent and one percent level at nine and ten weeks.

The effects of the menhaden meal and soybean oil meal were reversed in comparison to those of Experiment 1. In Experiment 2, menhaden meal exhibited no pigment-suppressing potency. However, soybean oil meal significantly lowered

TABLE 12. Summary of results of Experiment 2.

Group No.	Basal Diet	Modification	Ave. color scores				Ave. wt. 7 wks. gms.	Mortality %
			5 wks.	7 wks.	9 wks.	11 wks.		
1	7	None	7.0	7.2	-	-	585	10
2	7	15% meat scraps No. 1	5.1**	5.6**	-	-	516	10
3	7	15% menhaden meal No. 1	7.2	6.6	-	-	463	0
4	7	15% soybean oil meal No. 1	4.4**	5.8*	-	-	426	40
5	8	None	15.6	15.0	15.1	16.3	533	10
6	8	15% meat scraps No. 1	9.6◎◎	12.0	12.0◎	13.9◎	386	0

*Difference, when compared to group 1, statistically significant between the 5% and 1% levels.

**Difference, when compared to group 1, statistically significant beyond the 1% level.

◎Difference, when compared to group 5, statistically significant between the 5% and 1% levels.

◎◎Difference, when compared to group 5, statistically significant beyond the 1% level.

shank color score beyond the one percent level at five weeks and between the five percent and one percent levels at seven weeks.

Experiment 3. The results of Experiments 1 and 2 had indicated that perhaps the effect of the pigment-suppression observed therein was at a maximum at about five weeks and might disappear later. Too, the diets used in Experiments 1 and 2 were not practical diets. Therefore, Experiment 3 was designed to test the effect of these various protein supplements in practical rations and to study the relation of this phenomena to age.

A summary of the results of Experiment 3 is presented in Table 13. Study of this data reveals that inclusion of menhaden meal and meat scraps and a combination of these two products as sources of protein resulted in suppression of pigmentation in the shanks. When 19 percent of meat scrap was incorporated into the basal diet, pigmentation of the shanks was suppressed to an extent that was statistically significant beyond the one percent level at four, five and nine weeks of age. At ten weeks of age this difference was no longer significant beyond the one percent level but was significant between the five percent and one percent level.

The shank color score of the group that received 15 percent of menhaden meal in their diet was lower than that of the controls each time they were measured. These differences were statistically significant beyond the one percent level at five weeks and between the five percent and

TABLE 13. Summary of

Group	Protein Supplementation	Average color scores			
		4 wks.	5 wks.	9 wks.	10 wks.
1	15% soybean oil meal No. 1 13% dried skimmilk	11.3	11.9	10.7	10.7
2	15% soybean oil meal No. 1 13% dried buttermilk	11.8	10.7	12.0	12.2**
3	19% meat scraps No. 1	7.2*	7.7*	7.7*	9.0*
4	15% menhaden meal No. 1	10.0	9.9*	9.0	9.3*
5	7.5% meat scrap No. 1 6.0% menhaden meal No. 1 7.5% dried skimmilk	9.2*	10.7	8.8*	10.0
6	10% menhaden meal No. 1 13% dried skimmilk	12.1	12.7	11.4	10.5
7	15% soybean oil meal No. 1 2% menhaden meal No. 1 10% dried skimmilk	13.5**	12.2	11.3	12.3**
8	15% soybean oil meal No. 1 5% menhaden meal No. 1 5% dried skimmilk	10.2	10.3*	9.8	10.3

@Least significant difference in shank color

@@Pullorum disease diagnosed in chickens of this

*Difference, when compared with group 1, statis-

**Difference, when compared with group 1, statis-

results of Experiment 3.

Least significant differences@								Ave. wt.	Mortality to 10 wks. @@
4 weeks		5 weeks		9 weeks		10 weeks		10 weeks	
5%	1%	5%	1%	5%	1%	5%	1%	gms.	%
-	-	-	-	-	-	-	-	1019	32
1.48	1.96	1.29	1.70	1.58	2.09	1.09	1.44	967	24
1.69	2.20	1.47	1.94	1.89	2.50	1.36	1.79	854	68
1.57	2.07	1.38	1.82	1.79	2.36	1.19	1.58	830	52
1.41	1.86	1.22	1.62	1.50	1.99	1.00	1.33	963	4
1.42	1.88	1.24	1.63	1.50	1.99	1.02	1.35	935	4
1.48	1.96	1.29	1.70	1.56	2.07	1.05	1.38	1008	20
1.48	1.96	1.32	1.74	1.60	2.12	1.07	1.42	1054	28

score between group 1 and the respective groups.

experiment.

tically significant between the 5% and 1% levels.

tically significant beyond the 1% level.

one percent levels at ten weeks.

The results, when 7.5 percent of meat scraps and 6.0 percent of menhaden meal were included in the diet were very similar to those obtained when 15 percent of menhaden meal was included. There were relatively large differences at the time of each measurement. These differences were statistically significant beyond the one percent level at four weeks only and between the five percent and one percent levels at nine weeks only. Inclusion of five and ten percent of menhaden meal in the diet was without significant effect on shank color except that the shank color score of the five percent menhaden meal group was significantly lower between the five percent and one percent levels than that of the control group at five weeks.

The results when dried buttermilk was substituted for dried skimmilk are rather inconclusive. At four, nine and ten weeks the shank color score of the dried buttermilk group was greater than that of the group that had received the dried skimmilk, being statistically significant beyond the one percent level at ten weeks. At five weeks the reverse was true, the shank color score of the dried skimmilk group being higher than that of the dried buttermilk group, the difference closely approaching significance at the five percent level.

The inclusion of only two percent of menhaden meal in the diet increased the shank pigment score at each reading when compared to the control group. This increase was

statistically significant beyond the one percent level at four and ten weeks.

The data of this experiment indicate that the pigment-suppressing effect of the feedstuffs under study might become less apparent as the length of the experimental period is increased. There was no significant difference between the control group and group 2 until the tenth week. Between the control group and groups 3, 4, 5 and 6 the differences were not so highly significant at the close of the experiment as at four and/or five weeks but the difference between the control group and group 7 was significant beyond the 1 percent level at both four and ten weeks. In this respect, it is of interest to note that groups 3, 4 and 5 are highly comparable to some groups of Experiments 1 and 2, the results of which had indicated the possibility that this effect might not persist to ten weeks of age. Despite this decreasing effect as the length of the experimental period increases, the fact that significant differences do exist at ten weeks justifies study and consideration of the practical significance of this data.

Experiment 4. Experiments 1, 2 and 3 had shown that under certain circumstances menhaden meal, meat scraps and soybean oil meal suppressed shank pigmentation in growing chickens. The results of Experiment 3 had indicated that this suppression of pigmentation, although greatest at four or five weeks, was manifested in chickens up to ten weeks of age. In view of these results, it was felt that further

study on the distribution of this pigment-suppressing factor was warranted. Therefore, Experiment 4 was conducted to test more samples of these feedstuffs. Different lots of the same brand of meat scraps, menhaden meal and soybean oil meal had been used in Experiments 1, 2 and 3. Included in Experiment 4 were other lots of the same brands of menhaden meal and soybean oil meal used in Experiments 1, 2 and 3 and two samples of meat scraps No. 1. One of these samples of meat scraps No. 1 (groups 4 and 8) was identical with that used in Experiment 2 and had been stored at room temperature since the beginning of Experiment 2. The other (group 5) had been stored under refrigeration since the beginning of Experiment 2. In addition to these groups, a new sample each of menhaden meal and meat scraps and the supplementary effect of meat scraps No. 1 and soybean oil meal No. 1 were tested in Experiment 4.

The results of Experiment 4 are summarized in Table 14. The results are in general agreement with those of the previous experiments.

Again meat scraps No. 1 suppressed the shank color score significantly beyond the one percent level in comparison to the control group. In comparison to the results from Experiment 2, the data would indicate that storage, at either room temperature or under refrigeration, was without effect upon the pigment-suppressing properties of this sample.

Menhaden meal No. 1, as in Experiments 1 and 3 but in contrast to its effect in Experiment 2, significantly sup-

TABLE 14. Summary of results of Experiment 4.

Group	Basal Diet	Supplement to basal	Ave. color scores		Least significant differences@@				Ave. wt. 6 wks. gms.	Mortality to 6 weeks %
					4 weeks		6 weeks			
			4 wks.	6 wks.	5%	1%	5%	1%		
1	S	None	12.4	11.7	-	-	-	-	260	32
2	S	15% menhaden meal No. 1	9.5**	9.1**	1.51	2.00	1.17	1.55	303	24
3	S	15% menhaden meal No. 2	8.9**	9.5**	1.55	2.05	1.21	1.60	339	36
4	S	15% meat scraps No. 1	10.0**	9.9**	1.51	2.00	1.16	1.53	331	20
5	S	15% meat scraps No. 1@	9.6**	8.9**	1.50	1.97	1.15	1.51	320	20
6	S	15% meat scraps No. 2	9.0**	9.4**	1.55	2.05	1.59	2.10	284	72
7	S	15% soybean oil meal No. 1	12.1	10.9	1.50	1.97	1.17	1.55	368	24
8	S	(7.5% meat scraps No. 1 + 7.5% soybean oil meal No. 1)	10.6*	9.2**	1.62	2.14	1.41	1.86	301	60

@Identical to meat scrap No. 1 used in Experiment 2 and group 4 of this experiment except that it had been stored under refrigeration during the period between Experiments 2 and 4.

@@Least significant differences in shank color score between group 1 and the respective groups.

*Difference, when compared with group 1, statistically significant between the 5% and 1% level.

**Difference when compared to group 1, statistically significant beyond the 1% level.

pressed shank pigmentation.

Soybean oil meal No. 1, as in Experiment 1 but in contrast to its effect in Experiment 2, was without significant effect on the shank color score.

The two new products, menhaden meal No. 2 and meat scraps No. 2, suppressed shank pigmentation to a degree that was statistically significant beyond the one percent level at both four and six weeks.

As the sample of soybean oil meal exerted no effect on shank pigmentation, the suppression observed in group 8 of Experiment 4 as a result of inclusion of 7.5 percent of meat scraps No. 1 and 7.5 percent of soybean oil meal No. 1 are surprising. The shank color score in this group was statistically significant beyond the one percent level and was practically equal to that of group 4, which had received 15 percent of meat scraps No. 1.

Experiment 5. Experiment 5 was designed to test the pigment-suppressing properties of the ether soluble fraction of meat scraps No. 1. The results of this experiment are summarized in Table 15 and indicate that the cause of the pigment-suppression is not soluble in ether.

Fifteen percent of meat scraps No. 1 was included in the diets of two groups in Experiment 5. The color score of these two groups was lower than that of the positive control group, these differences being statistically significant beyond the one percent level.

The shank color score of group 4, which had received

[redacted]

TABLE 15. Summary of results of Experiment 5.

Group	Basal Diet	Modifications	Ave. color scores 5 wks.	Least significant differences at 5 wks.@@ 5%	1%	Ave. wt. 5 wks. gms.	Mortality to 5 wks. %
1	8A	None	12.5	-	-	172	16
2	8A	15% meat scraps No. 1	7.9**	1.84	2.43	143	56
3	8A	Ether extract of meat scraps No. 1@	12.7	1.45	1.92	166	20
4	8A	Residue of ether extraction@	8.6**	1.60	2.12	135	44
5	8A	15% meat scraps No. 1	9.0**	1.92	2.54	117	64
6	8A	Non-saponifiable fraction@	10.6*	1.63	2.17	157	48
7	8A	Saponifiable fraction@	11.4	1.63	2.17	140	48

@These fractions supplemented at a level equivalent to 30% of the original product with the exception of the residue of ether extraction. The residue of ether extraction, due to its high protein content, was fed at a level equivalent to 15% of the original product.

@@Least significant difference between group 1 and the respective groups.

*Difference, when compared with group 1, statistically significant between the 5% and 1% levels.

**Difference when compared with group 1, statistically significant beyond the 1% level.

[redacted]

the residue of ether extraction equivalent to 15 percent of meat scraps No. 1, closely approximated those of the groups that had been fed 15 percent of the original product and was significantly lower than that of the positive controls beyond the one percent level.

As was to be expected, the ether extract was without significant effect on the shank color score. However, the non-saponifiable fraction thereof did suppress pigmentation of the shanks to an extent that was statistically significant between the five percent and one percent level. The saponifiable fraction also suppressed pigmentation of the shanks somewhat but not to an extent that was statistically significant.

Experiment 6. Experiment 6 was designed to further study the distribution of the pigment-suppressing factor and determine if any relation existed between the mineral component of meat scraps No. 1 and its pigment-suppressing property.

The results of Experiment 6 are presented in Table 16. Unfortunately, the lot of meat scrap No. 1 used in this experiment did not significantly suppress the pigmentation of the shanks. This was the fifth lot of this product used and the first to fail to significantly suppress the shank color score. Since the original product failed to significantly suppress shank pigmentation, as was to be expected the ash thereof also failed to significantly affect shank pigmentation. However, at four weeks the shank color score of the

[redacted]

TABLE 16. Summary of results of Experiment 6.

Group	Supplementation to basal diet	Ave. color scores		Least significant differences@@				Wt. at 5 wks. gms.	Mortality to 5 wks. %
				4 wks.		5 wks.			
		4 wks.	5 wks.	5%	1%	5%	1%		
1	Positive control	9.00	11.7	-	-	-	-	302	8
2	15% meat scraps No. 1	8.00	11.4	1.40	1.85	1.35	1.79	288	4
3	15% meat scraps No. 3	8.10	9.7**	1.40	1.85	1.35	1.79	279	4
4	15% blood meal No. 1	7.70	9.5**	1.40	1.85	1.35	1.79	255	4
5	Ash meat scraps No. 1	10.1	11.7	1.37	1.82	1.32	1.75	236	0
6	Ferric ammonium sulfate@	9.2	11.4	1.40	1.85	1.36	1.80	296	12

@Ferric ammonium sulfate sufficient to supply iron equivalent to that contained in 15 percent of meat scraps No. 1.

@@Least significant differences between group 1 and the respective groups.

**Difference, when compared to group 1, statistically significant beyond the 1% level.

[redacted]

group fed the ash of meat scraps No. 1 was significantly greater beyond the one percent level than that of the group fed the meat scraps. At five weeks the shank color score of the former was slightly higher than that of the latter but the difference was no longer statistically significant.

The two new products tested, meat scraps No. 3 and blood meal No. 1, significantly depressed the shank color score beyond the one percent level.

Inclusion of ferric ammonium sulfate in the diet at a level that furnished the same quantity of iron as 15 percent of meat scraps No. 1 was without effect on the shank color score.

Experiment 7. The results of Experiment 7 are summarized in Table 17. This experiment was designed to isolate new sources of pigment-suppressing potent feedstuffs. Also included were groups to test the pigment-suppressing properties of cod liver oil and the supplementary effect of the cod liver oil and two of the other products tested.

Of the three feedstuffs tested, two proved to possess pigment-suppressing potency. The meat and bone scraps No. 1 and fish meal No. 3 significantly suppressed the shank color score, the former between the five percent and one percent level but very close to the one percent level and the latter beyond the one percent level. The fish meal base vitamin carrier was without effect on the shank color score.

Inclusion of three percent of cod liver oil in the basal diet resulted in a lowering of the shank color score,

TABLE 17. Summary of results of Experiment 7.

Group	Modifications	Ave. color scores 5 wks.	Least significant differences@ 5%	Least significant differences@ 1%	Tot. 5 wks. gms.	Mortality to 5 wks. %
1	None	3.00	-	-	245	0
2	15% meat and bone scraps No. 1	2.33*	.52	.69	336	6
3	15% fish meal base vitamin carrier	3.00	.51	.68	393	0
4	15% fish meal No. 3	2.19**	.51	.68	379	0
5	3% cod liver oil	2.20**	.52	.69	257	6
6	As 2 + 3% cod liver oil	2.20**	.57	.76	251	30
7	As 4 + 3% cod liver oil	1.53**	.52	.69	361	0

@Least significant difference between group 1 and the respective groups.

*Difference, when compared to group 1, statistically significant between the 5% and 1% level.

**Difference, when compared to group 1, statistically significant beyond the 1% level.

significant beyond the one percent level. When three percent of cod liver oil, in addition to fish meal No. 3, was included in the diet the suppression of pigmentation was much greater than when either was included alone. However, there was no supplementary effect between the cod liver oil and meat and bone scraps No. 1. The shank color score of group 5, which received the cod liver oil alone, was identical with that of group 6, the diet of which contained 3 percent of cod liver oil and 15 percent of meat and bone scraps No. 1.

Experiment 8. This experiment was conducted to ascertain the effect of heat on the pigment-suppressing properties of meat and bone scraps and fish meal. The results are presented in Tables 18 and 19.

The lot of meat and bone scraps No. 1 used in this experiment was without significant effect on the shank color score as were the dry-heated and autoclaved samples of this product.

Fish meal No. 3 suppressed pigmentation of the shanks to a degree that was significant beyond the one percent level. Dry-heated fish meal No. 3 did not surpress pigmentation to the extent that the untreated product did. However, the suppression was still significant beyond the one percent level. As shown in Table 19 the difference between the untreated group and the dry-heat treated group was not significant. The sample of fish meal No. 3 that had been autoclaved did not suppress pigmentation to an extent that

[redacted]

TABLE 18. Summary of results of Experiment 8.

Groups	Supplement to basal diet	Ave. color scores	Least significant differences@ 5%	1%	Ave. wt. 5 wks. gms.	Mortality %
10 & 13	None	5.66	-	-	260	6.3
6 & 14	Meat and bone scraps No. 1	5.50	1.20	1.59	201	25.0
4 & 7	Dry-heated meat and bone scraps No. 1	5.88	1.36	1.80	177	50.0
1 & 3	Autoclaved meat and bone scraps No. 1	5.07	1.15	1.53	213	12.5
11 & 12	Fish meal No. 3	3.15**	1.18	1.56	378	12.5
8 & 9	Dry-heated fish meal No. 1	4.00**	1.15	1.53	352	12.5
2 & 5	Autoclaved fish meal No. 1	4.54	1.18	1.56	309	18.8

@Least significant differences between groups 10 and 13 and the respective groups.

**Difference, when compared to group 1, statistically significant beyond the 1% level.

[redacted]

TABLE 19. Differences and least significant differences between groups receiving heat treated and untreated products in Experiment 8.

Treatment	Fish meal No. 3				Meat and bone scraps No. 1			
	Groups	Difference in shank color score between treated and untreated groups	Least significant difference		Groups	Difference in shank color score between treated and untreated groups	Least significant difference	
			5%	1%			5%	1%
Dry-heated	8 & 9	+0.85	1.19	1.58	4 & 7	+0.38	1.42	1.88
Autoclaved	2 & 5	+1.39*	1.22	1.61	1 & 3	-0.43	1.22	1.61

*Significantly greater than that of the untreated group between the 5% and 1% levels.

was statistically significant when compared to the positive control group. As shown in Table 19 the shank color score of groups 2 and 5, which had been fed the autoclaved fish meal, was significantly greater between the five percent and one percent levels than that of groups 11 and 12, which received the untreated fish meal in their diet.

Experiment 8 was conducted in duplicate. However, the data of the duplicate groups has been presented as a single group. Analysis of variance for replication gave an almost unbelievably low F value of 0.534. An F value of 253 at the five percent level and of 6323 at the one percent level is necessary for significance for the degrees of freedom involved. In view of the small amount of the total variation due to variation between the duplicates, this procedure is justified.

Experiment 9. This experiment was designed to investigate the possibility of a relationship between the pigment-suppressing property of meat and bone scraps No. 1 and the carotene destroying power of meat and bone scraps reported by Sherwood and Fraps (53). A summary of the results, which were essentially negative, is presented in Table 20.

A comparison of group 7, fed the vitamin A deficient basal diet, and group 5, fed the vitamin A deficient basal diet plus 20 percent of meat and bone scraps No. 1, reveals the essentially negative character of the results. At five weeks of age the total mortality in group 7 had reached 93.4 percent with a total mortality of 73.4 percent being

[redacted]

TABLE 20. Summary of results of Experiment 9.

Groups	Supplement to vitamin A deficient diet	Mortality %	Mortality attributed to vitamin A deficiency %	Ave. wt. 5 wks. gms.
7	None	93.4	73.4	150
1	0.5% alfalfa leaf meal	12.5	12.5	156
13	0.5% alfalfa leaf meal	37.5	12.5	183
8	1.0% alfalfa leaf meal	12.5	12.5	175
11	1.0% alfalfa leaf meal	0.0	0.0	151
2	1.5% alfalfa leaf meal	12.5	0.0	194
12	1.5% alfalfa leaf meal	0.0	0.0	198
6	2.0% alfalfa leaf meal	12.5	0.0	186
14	2.0% alfalfa leaf meal	12.5	0.0	205
5	20% meat and bone scrap No. 1	60.0	46.7	190
9	As 5 + 0.5% alfalfa leaf meal	12.5	0.0	256
17	As 5 + 0.5% alfalfa leaf meal	25.0	12.5	227
3	As 5 + 1.0% alfalfa leaf meal	25.0	12.5	271
15	As 5 + 1.0% alfalfa leaf meal	0.0	0.0	263
4	As 5 + 1.5% alfalfa leaf meal	0.0	0.0	269
18	As 5 + 1.5% alfalfa leaf meal	0.0	0.0	296
10	As 5 + 2.0% alfalfa leaf meal	0.0	0.0	207
16	As 5 + 2.0% alfalfa leaf meal	0.0	0.0	293

[redacted]

attributed to vitamin A deficiency. The total mortality in group 5 at the termination of the experiment amounted to 60.0 percent and the total mortality attributed to vitamin A deficiency amounted to 46.7 percent. The one chicken remaining in group 7 at the termination of this experiment exhibited definite symptoms of vitamin A deficiency as did all those remaining in group 5.

Due to the fact that the pigment content of most highly pigmented diet was so low, the color of the shanks of the positive control chickens that had been fed this diet was a minimum value. When attempts were made to measure the shank color of groups 6 and 14 and groups 10 and 16 for comparison the values for all groups were a minimum. It was impossible to select from either of these groups any chicken that had measurably darker colored shanks than those of groups 5 and 7, which had received practically pigment-free diets.

Experiment 10. Experiment 10 was an expansion of Experiment 8. The same sample of fish meal No. 3 and a sample of meat and bone scraps No. 1 different to that used in Experiment 8 were tested in Experiment 10. Also included were one untested sample each of fish meal and meat scraps and autoclaved samples of each of these four products. The results are presented in Tables 21 and 22.

The results of the autoclaving of the fish meal No. 3 are in close agreement with those of Experiment 8. The shank color of group 6, which had received autoclaved fish meal No. 3 in its diet, was significantly greater beyond the one

TABLE 21. Summary of results of Experiment 10.

Group	Supplement to basal diet	Ave. Color Score	Least significant differences@ 5%	Least significant differences@ 1%	Ave. wt. 4 wks. gms.	Mortality 4 wks. %
3	None	5.53	-	-	199	0
1	Fish meal No. 3	3.20**	.68	.89	248	6
6	Autoclaved fish meal No. 3	4.46**	.70	.93	183	30
5	Fish meal No. 4	4.06**	.66	.86	246	0
9	Autoclaved fish meal No. 4	4.06**	.67	.88	192	6
8	Meat and bone scraps No. 1	4.25**	.67	.88	124	6
2	Autoclaved meat and bone scraps No. 1	4.71*	.66	.86	151	0
7	Meat scraps No. 4	4.75*	.67	.88	220	6
4	Autoclaved meat scraps No. 4	4.53**	.66	.86	162	0

@Least significant difference between group 3 and the respective groups.

*Difference, when compared to group 3, statistically significant between the 5% and 1% levels.

**Difference, when compared to group 3, statistically significant beyond the 1% level.

TABLE 22. Differences and least significant differences between groups receiving the untreated and autoclaved feedstuffs tested.

Groups	Feedstuff	Difference in color score	Least significant difference	
			5%	1%
1 and 6	Fish meal No. 3	+1.26**	.73	.95
5 and 9	Fish meal No. 4	0.00	.67	.88
8 and 2	Meat and bone scraps No. 1	+0.56	.67	.88
7 and 4	Meat scraps No. 4	-0.22	.67	.88

**Statistically significant beyond the 1% level.

percent level than that of group 1, its control, but significantly smaller beyond the one percent level than that of the positive control.

Autoclaving was without significant effect on the pigment suppressing power of any of the other feedstuffs tested as shown in Table 22.

Growth and mortality. In experimentation of this nature it is highly desirable that the experimental diets support normal growth and livability. Many of the diets used in these experiments do not meet these requirements. Efforts to design a ration that met these requirements and was at the same time satisfactory for measurement of the pigment-suppressing properties of the feedstuffs under test met with little success. In the formulation of diets, the factor of prime importance was that they contain no variable component that might influence the color of the shanks. Included in this category are feedstuffs that contain xanthophyll and others that might be sources of the pigment-suppressing factor.

As growth and livability were not of prime consideration in these experiments, discussion on these two subjects was omitted from the presentation of the results of the individual experiments. There was considerable variation in growth and mortality of the chickens on the various rations both within and between experiments. The rate of growth on basal diet three, Experiment 1 was very slow and livability was excellent. However, when this diet was supplemented with

fish meal No. 1, the rate of growth was appreciably accelerated but mortality was very high. Supplementation with meat scraps No. 1 had little effect on the rate of growth but did greatly increase the mortality.

The growth rate and livability on basal diets seven and eight, Experiment 2, was satisfactory but supplementation of these diets with meat scraps No. 1, menhaden meal No. 1 and soybean oil meal No. 1 decreased the rate of growth. Livability was excellent in all supplemented groups except the soybean oil meal, in which the mortality amounted to 40 percent.

The results obtained using diet eight in Experiment 4 are in complete contrast with those obtained on this diet in Experiment 2. In Experiment 2 the rate of growth and livability had been excellent on this diet. In Experiment 4 both were far from satisfactory. Too, all supplements used increased the rate of gain while in Experiment 2 the weight of the meat scraps supplemented group was depressed in comparison to the positive control group. Mortality in all groups was considered excessive, ranging from 20 percent to 72 percent. There was no cause to which this mortality could be attributed.

The results of Experiment 5, Table 15, as far as rate of growth and mortality are concerned, are very similar to those of Experiment 4, Table 14. Diet 6 and a slight modification thereof were used in a total of three experiments. In only one of these, Experiment 2, did it produce

normal growth and livability.

As a result of the near disaster to this work due to the high mortality and poor growth on diets 8 and 8A used in Experiments 4 and 5, respectively, this ration was abandoned and a diet closely approaching a practical diet was adopted for use in Experiment 6. As the results given in Table 16 indicate this ration supported excellent growth and livability in this experiment was good. However, the ration was unsatisfactory in that it was not so sensitive to the pigment-suppressing factor. The shank color score of the positive control group was approximately the same as that of the positive control group on diet 8. However, the supplements did not produce equal suppression of the shank color score. This interpretation was not tested experimentally and it may well have been that the supplements used did not possess the pigment-suppressing power of those used previously.

The basal diets used in Experiments 7, 8 and 10 produced satisfactory growth and livability. They were also very sensitive to sources of the pigment-suppressing supplements as indicated by the relatively large differences between shank color score of the positive control and supplemented groups.

The diets used in Experiment 3, the results of which are summarized in Table 13, were considered practical diets. Growth on these diets was satisfactory, however, mortality was very high. A portion of the mortality in this experi-

ment is explained by an invasion of pullorum disease. It is doubtful if this factor accounts for the differences in mortality observed between groups.

DISCUSSION

It was the purpose of this study to determine if components of the diet of the growing chickens, other than the concentration of xanthophyll in the diet, influenced the intensity of the yellow color of the shanks of breeds of chickens that deposit yellow pigment in their shanks. Since Palmer (35) demonstrated that the yellow pigment of chickens was xanthophyll, and identical to that of plants, it has been generally assumed that the intensity of the color of the visible skin parts of growing chickens was controlled exclusively by the amount of xanthophyll in the diet. The data presented here, and the results of other research work published during the period that this work has been in progress, render this theory no longer tenable.

The results of all experimental trials using untreated feedstuffs are summarized in Table 23. A total of 30 samples of commonly used feedstuffs were tested for their pigment-suppressing power. Twenty-four of these samples significantly depressed the shank color score and six failed to do so. In general animal protein supplements were the worst offenders.

Nine samples of four brands of fish meal and one of a fish meal base vitamin carrier were tested. Eight of the

TABLE 23. Summary of results of feedstuffs tested in Experiments 1 to 10, inclusive.

Sample	No. of times tested	No. of times positive	No. of times negative
Soybean oil meal No. 1	3	1	2
Menhaden meal No. 1	4	3	1
Menhaden meal No. 2	1	1	
Fish meal No. 3	3	3	
Fish meal No. 4	1	1	
Fish meal base vitamin carrier	1	1	
Meat scraps No. 1	9	8	1
Meat scraps No. 2	1	1	
Meat scraps No. 3	1	1	
Meat scraps No. 4	1	1	
Meat and bone scraps No. 1	3	2	1
Wheat flour middlings	1		1
Blood meal No. 1	1	1	

fish meal samples significantly lowered the shank color score. One sample of fish meal and the sample of fish meal base vitamin carrier were without significant effect on this entity.

Of 15 samples of meat scraps and meat and bone scraps of five different brands tested, 13 samples significantly depressed the shank color score.

One sample of blood meal was tested with positive results.

Two feedstuffs of plant origin were tested. They were three samples of one brand of soybean oil meal and one sample of wheat flour middlings. The soybean oil meal significantly lowered the shank color score in only one of the three trials. If the wheat flour middlings exerted any effect it was pigment-promoting in the one experimental trial conducted.

The pigment-suppressing potency of individual feedstuff varied with different lots used in different experiments. Menhaden meal No. 1 significantly lowered the shank color score in three of four experimental trials and was without effect in one experimental trial. The same situation exists with regards to the results obtained with meat scraps No. 1 and meat and bone scraps No. 1. In eight of nine tests the former was positive in suppressing pigmentation of the shanks and was negative in one test. The latter gave positive results twice and negative results once in three experimental trials.

No satisfactory explanation for the failure of different

samples of the same brand of a feedstuff to consistently suppress the pigmentation of the shanks is indicated by the experimental results. The only test in which meat scraps No. 1 failed to significantly suppress pigmentation was in Experiment 6, Table 16. The ration used in this experiment had not been used previously and was not used thereafter.

The one positive result with soybean oil meal No. 1 and the one negative result with menhaden meal No. 1 were also observed on rations that for some reason were not tested with these products again. The soybean oil meal was negative on diet three, Experiment 1 and positive on diet seven, Experiment 2. The reverse was true of menhaden meal No. 1. In Experiment 1 menhaden meal No. 1 gave positive results and in Experiment 2 negative results. Diets three and seven were not used again, but on diet eight, Experiment 4, the results with these two products were the same as those in Experiment 1.

These results are most easily explained on the assumption that the pigment-suppressing properties of different samples are due to different factors and that some component of the different diets interferes with the mechanism of pigment-suppression involved. The effect of heat-treatment on various pigment-suppressing feedstuffs is cited as evidence in support of the possibility of different factors in different samples of feedstuffs being responsible for the pigment-suppressing properties thereof. However, results with meat and bone scraps No. 1 are in complete conflict with

this explanation. The same basal ration was used in Experiments 8 and 10, yet in Experiment 8, supplementation with meat and bone scraps No. 1, groups 6 and 14 in Table 18, was without effect on the color of the shanks while in Experiment 10 supplementation with meat and bone scraps No. 1, groups 2 and 8, Table 21, resulted in the lowering of the shank color score that was highly significant. In view of the latter results, the most logical assumption is that the constituents that go into the manufacture of the product or the method of manufacture, or both, affect the pigment-suppressing potency of the product produced.

The results obtained on the suppression of pigment in the shanks of chickens are interestingly similar to those reported by Sherwood and Fraps (53) on the carotene-destroying power of certain animal protein supplements. These authors reported that certain samples of meat scraps, when incorporated into the diet, destroyed the carotene in the diet. In their experiments one out of five lots of meat scraps tested proved to possess the power to destroy carotene. In the experiments reported here the percentage of samples tested that exhibited the anti-xanthophyll effect was much greater. Sherwood and Fraps (53) also observed that the carotene destroying power of a given feed varied from time to time. They did not state whether they referred to the same sample of feed or to different samples of the same brand. Since they specified a given feed it is assumed that they mean the former. No variation in the anti-xantho-

phyll effect between experiments with the same sample of feed was observed in these experiments, but different samples of the same brand of a given feedstuff did differ in their anti-xanthophyll effect.

Due to the fact carotene and the xanthophylls are so very similar in their chemical structure it is within the realm of possibility that Sherwood and Fraps (53) were working with the same identity as these were designed to study. The results of Experiment 9, which was designed to test this possible relationship, were essentially negative through faulty design. The same sample of meat and bone scraps No. 1 were used in Experiments 8 and 9. In Experiment 8, which was not completed prior to the beginning of Experiment 9, this sample of meat and bone scraps exhibited no pigment-suppressing potency. In view of this result, if the experiments of Sherwood and Fraps (53) were concerned with the same factor as these experiments, no destruction of carotene would be expected. That there was no appreciable destruction is evidenced by a comparison of groups 7, 1 and 13, and 8 and 11 to groups 5, 9 and 17, and 3 and 15 respectively, in Table 20. Mortality to five weeks was greater in the negative control, group 7, than in the negative control meat and bone scraps No. 1, group 5. Also, a higher percentage of the mortality in group 7 than in group 5 was attributed to vitamin A deficiency. Undoubtedly this difference would have become decreasingly smaller had the experiment been continued to a point where the total mortality in group

5 equalled that in group 7. At the termination of the experiment all birds that remained in group 5 exhibited definite symptoms of vitamin A deficiency. Had the experiment been continued until these birds died, a greater percentage of these deaths, than of the former ones, would have undoubtedly been attributed to vitamin A deficiency. Theoretically, the diets of groups 7 and 5 of Experiment 9 contained no carotene and, if so, it is logical to ask what information does the results of these groups yield that is pertinent to the question under discussion. Actually the results yield no direct information but certain inferences may be drawn therefrom. The fact that the mortality was not so great in group 5 as in group 7 would indicate that the meat scraps probably contained a small amount of vitamin A. Too, there was no appreciable difference in mortality attributed to vitamin A deficiency and the appearance of the external symptoms of vitamin A deficiency between the groups that had received the vitamin A basal diet supplemented with 0.5 percent and 1 percent alfalfa leaf meal and those that had received the meat and bone scraps No. 1 diet supplemented with the same amount of alfalfa leaf meal. The shanks of the chickens that had received the most highly pigmented diets in Experiment 9 were not measurably darker in color, with the methods used, than those on the pigment-free diets. These facts led to the conclusion that the hoped for results of this experiment would not materialize nor would it yield any other pertinent information. Therefore, it was discon-

tinued.

The report of Rubin, Bird and De Volt (48) and Jungherr (24) on the rather widespread avitaminosis A in commercial poultry flocks, indicates the necessity for further study on the possible relation of the carotene destroying power and the anti-xanthophyll effect of feedstuffs. It is felt that irrefutable demonstration of a definite relationship between the anti-xanthophyll effect and carotene destroying power of feedstuffs would partially explain the reported widespread avitaminosis A in commercial poultry enterprises. Too, if a definite relationship was found to exist between these two properties it would serve as an aid in formulating practical mashes. The rather extensive tests reported here, conducted over a period of years, indicates that the anti-xanthophyll effect of meat scraps and fish meals is rather widespread.

The report of Hammond and Harshaw (16) that cod liver oil contained a factor that suppressed the pigmentation of the shanks of growing chickens suggested the possibility that the pigment-suppressing effect observed in experiments conducted previous to their report, might be contained in the fat-soluble portion of those feedstuffs that exhibited pigment-suppressing potency. That the effects observed by these authors is not identical to that reported here is evidenced by the results of Experiment 5 and reports that have appeared in the literature since the original report of Hammond and Harshaw (16). The pigment-suppressing potency

of meat and bone scraps No. 1 was unaffected by extraction with ether for 48 hours. The residue of ether extraction, group 4 of Experiment 5 summarized in Table 15, suppressed the shank color score equal to that of the original product. When the ether extract of this product was included in the diet, group 3 of the same experiment, at a level equivalent to 30 percent of the original product, there was no significant effect on the shank color score when compared to that of the positive controls.

Surprisingly, the non-saponifiable fraction, when included in the diet at a level equivalent to 30 percent of the original product, suppressed shank pigmentation to a degree that was significant between the five percent and one percent levels. The saponifiable fraction also suppressed the pigmentation of the shank, but this suppression was not significant. This suppression of pigmentation by the saponifiable and non-saponifiable fractions of the ether extract was unexpected in view of the results of the group that received the residue and ether extract, and is difficult to explain. The shank color score of the group that had been fed the residue was very close to that of the negative control group, considered as evidence that the pigment-suppressing property of the original product had been unaffected by ether extraction.

The data from the group fed the ether extract are equally convincing that ether extraction was without effect on the pigment-suppressing properties of this sample. When

this fraction was included in the diet, at a level equivalent to 30 percent of the original sample, the shank color score was practically equal to that of the positive control group. Perhaps the most logical explanation is one based on the effect of the saponification and subsequent neutralization of the potassium hydroxide used. Too, the report by Maw and Nikolaiczuk (34) on the reduction of the carotenoid content of shank skin as a result of injection of one-half cubic centimeters of 15 percent ethyl alcohol, cannot be ignored as a possible explanation. The fat was saponified with alcoholic potassium-hydroxide and the alcohol removed by distillation under reduced pressure. Undoubtedly this distillation was not 100 percent effective. Whether the feeding of alcohol would produce results similar to those reported by Maw and Nikolaiczuk (34), as a result of the injection of alcohol is a matter of speculation. That a similar effect could result from oral administration of alcohol is certainly within the realm of possibility.

The results of Experiment 5 had definitely indicated that the pigment-suppressing properties of cod liver oil, as reported by Hammond and Harshaw (16), was not identical with the causative factor being studied in these experiments. Further proof of the non-identity of these two causes of suppression has been presented by Mattson and Deuel (33) and Rubin and Bird (47). Mattson and Deuel (33) demonstrated that vitamin A from shark liver oil decreased the carotenoid content of the liver and blood serum in growing

chickens. Rubin and Bird (47) repeated a portion of the work of Hammond and Harshaw (16) and obtained evidence that the pigment-suppressing effect of cod liver oil was due to its vitamin A content. This identification of the factor in cod liver oil responsible for the suppression of pigmentation in the shanks made further work on the comparison of the two factors unnecessary.

The pigment-suppressing potency of fish meal No. 3 was definitely heat-labile. In Experiment 8, the shank color score of groups 2 and 5, which had received the autoclaved fish meal No. 3 supplemented diet, was significantly higher between the five percent and one percent level than that of its control, groups 11 and 12. However, autoclaving at 15 pounds of pressure for four hours did not completely destroy the pigment-suppressing property of this sample as evidenced by a comparison of the shank color score of positive controls, group 10 and 13 in Table 18 to groups 2 and 5 of this same table. The difference between the positive controls and the autoclaved fish meal group was not significant but did closely approach significance at the five percent level. The results of Experiment 10 confirmed the heat-labile nature of the pigment-suppressing potency of fish meal No. 3 observed in Experiment 8. The shank color score of group 6, Table 21, which had received the autoclaved fish meal No. 3 diet was significantly greater than that of group 1, its control. This difference was statistically significant beyond the one percent level. The sample

used in this experiment was autoclaved for eight hours at 15 pounds of pressure. This treatment did not completely destroy the pigment suppressing potency of fish meal No. 3. In fact, the results indicate that the eight hour treatment was not so completely effective as was the four hour treatment. In Experiment 8, there was no significant difference between the shank color score of the positive controls and that of the group which had been fed autoclaved fish meal, but in Experiment 10, the difference in shank color score between the two comparable groups was significant beyond the one percent level.

In Experiment 8, subjecting the fish meal to a temperature of 76 degrees centigrade for a period of 48 hours, did not result in a significant effect on the shank color score.

The sample of meat and bone scraps No. 1 used in Experiment 8 failed to suppress the shank color score significantly. Therefore, it would not be expected that either the autoclaving or dry-heat treatment would result in significant effect on the pigment-suppressing property of this sample.

The two samples each of fish meal and of meat by-products used in Experiment 10 significantly suppressed the shank color score. However, autoclaving was effective in reducing the pigment-suppressing potency of only one sample, fish meal No. 3. The most plausible explanation for this observed difference is that the causative factor of pigment-suppression in fish meal No. 3 is different from that of fish meal No. 4, meat scraps No. 4, and meat and bone scraps

No. 1. Had the results of the autoclaving of the two samples of fish meal been similar, the relatively great differences in the raw material that goes into the manufacture of the fish meal and meat by-products could readily account for the presence of two or more causative factors.

This, of course, was not the true situation. However, the experimental evidence does suggest the existence of a heat-labile and a heat-stable pigment-suppressing factor. The fact that the heat-labile factor is not present in all samples could be due to differences in the manufacturing processes. If the raw material used in the manufacture of these products contained a heat-labile and heat-stable pigment-suppressing factor, then differences in heat applied during the dehydrating process would affect the pigment-suppressing potency of the end product. One manufacturing process might well apply sufficient heat to destroy the heat-labile factor while another would fail to do so. In this case, it would have to be assumed that fish meal No. 3 had been manufactured by a process that employed a comparatively low temperature. The fact that autoclaving of this sample for four hours, Experiment 8, was as effective as autoclaving eight hours, Experiment 10, in destroying its pigment-suppressing ability would indicate that a portion of the pigment-suppressing ability was relatively stable to heat.

The effect of heat treatment on fish meal No. 3 immediately suggests the possibility of destruction of xanthophyll due to the presence of an enzyme in this sample.

However, the possibility of an enzyme escaping destruction during the manufacturing process is remote. Deuel, Halliday, Hallman, Johnston and Miller (11) suggested that the depression of milk and blood carotene in cattle as a result of increased vitamin A intake was possibly due to development of a new enzyme system or increased activity of an enzyme system already present that was capable of destroying carotene. As evidence they cited the relatively slow development of the maximum effect and the continued manifestation of this effect after high vitamin A feeding was discontinued. Deuel, Hrubetz, Mattson, Morehouse and Richardson (13) suggested that if the development of a new enzyme system or increased acceleration of an enzyme system already present was responsible for the lowering of the carotene content of the milk and blood, as reported by Deuel, Halliday, Hallman, Johnston and Miller (11), then this enzyme system was not specific for carotene, but was also capable of destroying the xanthophylls, as high vitamin A intake exerted the same effect on the xanthophyll content of egg yolk, blood serum and liver of chickens. It is reiterated that the results reported here could not be due to vitamin A, but if the effect of vitamin A is due to the establishment of a new enzyme system or increased acceleration of an already present one, the effects observed in these experiments could be due to the same physiological mechanism. The results of these experiments indicate no explanation of the mode of action of the pigment-suppression. Too, the possibility of

destruction of the xanthophyll in the mixed ration cannot be ignored.

The results of these experiments could definitely explain the difficulty encountered in producing desirably colored yellow shanks in broilers. Admittedly the percentages of the animal protein supplements used in the work reported herein is higher than that generally recommended for practical mashes. However, it is not uncommon to encounter formula for starting mashes with recommended levels of fish meal and meat scraps amounting to ten percent of the total ration. Too, at least one sample of soybean oil meal tested suppressed shank pigmentation and the total percentage of soybean oil meal, fish meal and meat scraps in most practical growing mashes is greater than that used in these tests. In view of this situation it is highly probable that many of the failures of practical starting diets to produce optimum pigmentation could be explained by the results of these experiments. Too, the variation in the pigment-suppressing property of different samples of the same brand of feedstuffs serves as a possible explanation of the variation in pigmentation observed between different lots of broilers fed on different lots of feed mixed from the same formula.

SUMMARY

1. Evidence is presented that dietary factors, other than the amount of pigment in the diet, affects the

deposition of pigment in the shank skin of growing chickens.

2. The pigment-suppressing causative agent was not extracted by continuous extraction for 48 hours with ethyl ether. The residue of ether extraction possessed pigment-suppressing potency equal to that of the sample used for extraction.

3. The experimental results indicate the presence of a heat-labile and a heat-stable pigment-suppressing factor. The pigment-suppressing potency of one sample of fish meal was significantly decreased by autoclaving at 15 pounds of pressure for four or eight hours. Autoclaving for eight hours was without effect on the pigment-suppressing potency of one sample of fish meal and two samples of meat by-products.

4. Thirty samples of commonly used feedstuffs were tested for their pigment-suppressing potency. Twenty-four of these samples significantly lowered the shank color score and six were without effect on this entity.

5. The pigment-suppressing causative agent is widely distributed in animal protein supplements. Ten samples of protein supplements manufactured from fish by-products and 15 manufactured from meat by-products were tested for their pigment suppressing effect. Eight of the former and 13 of the latter were positive.

6. Four samples of feedstuffs of plant origin were tested. One sample of wheat flour middlings did not lower the shank color score. Of those samples of soybean oil meal

tested, only one significantly lowered the shank color score.

7. The pigment-suppressing potency of different samples of the same brand of a feedstuff varied among experiments. The results of these experiments indicate no explanation for these observed differences. It is felt that this variation could be of two sources. One, the differences in the raw products used in the manufacture and/or differences in the manufacturing process. The other possibility is that different components of the various diets might interfere with the pigment-suppressing mechanism of one feedstuff and not of another.

8. The results of these experiments offer a possible explanation of the failure of many relatively highly pigmented diets to produce optimum pigmentation of the shank skin in broilers and, also, serve as a basis for the explanation of variation in pigmentation of the shank skin observed between different lots of broilers which have been fed different lots of feed prepared from the same formula.

LITERATURE CITED

1. Almquist, H. J., G. MacKinney and E. Mecchi. The diet of the hens and the vitamin A potency of their eggs. Jour. Biol. Chem. 150:99-105. 1943.

2. Ball, R. F. The value of four characters used in culling ready-to-lay pullets. Poultry Sci. 24:216-225. 1945.

3. Barrows, H. R. Histological basis of shank colors in the domestic fowl. Report of the Maine Agric. Exp. Sta., Bul. 232:237-252. 1914.

4. Benjamin, E. W., and H. C. Pierce. Marketing poultry products. New York: John Wiley and Sons, Inc., 1937. 401 pp.

5. Bisbey, Bertha, Virginia Appleby, Adelia Wise and Sylvia Cover. The vitamins A and D activity of egg yolks of different color concentrations. Mo. Agric. Exp. Sta. Res. Bul. 205:1-32. 1934.

6. Bisbey, Bertha, Adelia Wise and H. L. Kempster. Influence of the rations of the hen upon the vitamin A and Riboflavin content of the eggs and tissues. Poultry Sci. 19:345. 1940. An abstract.

7. Bogert, M. F. Organic Chemistry edited by Henry Gilman. pp 1138-1219. New York: John Wiley and Sons, Inc. 1938.

8. Bohren, B. B., C. R. Thompson and C. W. Carrick. The transfer of carotenoid pigments to the egg yolks. Poultry Sci. 24:356-362. 1945.

9. Bolin, D. W., C. E. Lampman, and L. R. Berg. The influence of carotene intake as supplied by dehydrated alfalfa on the storage of vitamin A and pigments in the livers of young chicks. Poultry Sci. 22:348-353. 1943.

10. Brockmann, Hans and Otto Valker. The yellow pigment of the canary (Serinus Canaria Canaria) and the occurrence of carotenoids in birds. Zeit. f. Physiol. Chem. 224:193-215. 1934.

11. Deuel, H. J., Jr., N. Halliday, L. F. Hallman, C. Johnston and A. J. Miller. The production of high vitamin A milk by diet. Jour. Nutrition 22:303-313. 1941.

12. Deuel, H. J., Jr., L. F. Hallman, C. Johnston and F. Mattson. The effect of a high vitamin A intake on the blood and milk carotene of Holstein and Guernsey cows. Jour. Nutrition 23:567-579. 1942.

13. Deuel, Harry J., Jr., M. Caroline Hrubetz, Fred H. Mattson, Margaret G. Morehouse and Alan Richardson. Studies on carotenoid metabolism. IV. The effect of vitamin A intake on the carotenoid and vitamin A content of the eggs, liver, blood and body fat of hens. Jour. Nutrition 26:673-685. 1943.

14. Fisher, R. A. Statistical Methods for Research Workers. London: Oliver and Boyd, 1941. 344 pp.

15. Gillam, A. E. and I. M. Heilbron. Vitamin A active substances in egg yolk. Biochem. Jour. 29:1064-1067. 1935.

16. Hammond, John C. and Harold M. Harshaw. Some factors influencing shank and skin color in the growing chicken. Poultry Sci. 20:437-444. 1941.

17. Hammond, John C., David Miller and Donald Whitson. The effect of the diet of the hen on the shank color of her day old chicks. Poultry Sci. 21:525-527. 1942.

18. Heiman, V., and J. S. Carver. The yolk color index. U. S. Egg and Poultry Magazine 41:40-41. 1935.

19. Heiman, Victor and L. W. Tighe. Observations on the shank pigmentation of chicks. Poultry Sci. 21: 471-472. 1942. An abstract.

20. Heiman, Victor and L. W. Tighe. Observations on the shank pigmentation of chicks. Poultry Sci. 22:102-107. 1943.

21. Henderson, E. W., and H. L. Wilcke. Effect of ration on yolk color. Poultry Sci. 12:266-273. 1933.

22. Hollander, W. F., and R. D. Owen. The carotenoid nature of yellow pigment in the chicken iris. Poultry Sci. 18:385-387. 1939.

23. Hughes, J. S. and L. F. Payne. The relation of the carotenoid pigments of feed to the carotenoid pigments of egg yolk. Poultry Sci. 16:135-138. 1937.

24. Jungherr, Erwin. A-hypovitaminosis in commercial poultry flocks on basis of nasal histopathology. Poultry Sci. 24:112-119. 1945.

25. Karrar, P., K. Schopp and R. Morf. Plant pigment. XLII. The isomeric carotenes and their relationship to the growth-producing vitamin A. Helv. Chim. Acta. 15:1158-1165. 1932.

26. Kline, O. L., M. C. Schultze and E. B. Hart. Carotene and xanthophyll as sources of vitamin A for the growing chick. Jour. Biol. Chem. 97:83-91. 1932.

27. Kuhn, Richard and Hans Brockmann. Determination of carotenoids. Zeit. f. Physiol. Chem. 206:41-64. 1932.

28. Kuhn, Richard and Christoph Grundmann. The growth vitamin. V. Cryptoxanthin, a xanthophyll of the formula, $C_{40}H_{56}O$. Ber. Deutsch. Chem. 66:1746-1750. 1933.

29. Kuhn, Richard and Christoph Grundmann. The growth vitamin. VI. Cryptoxanthin from yellow corn. Ber. Deutsch. Chem. 67:593-595. 1934.

30. Kuhn, Richard and Alexander Smakula. Spectrophotometric analysis of the yellow pigments of egg yolk. Zeit. f. Physiol. Chem. 197:161-166. 1931.

31. Kuhn, Richard, Alfred Winterstein and Edgar Lederer. The xanthophylls. Zeit. f. Physiol. Chem. 197: 141-160. 1931.

32. Mattikow, M. A critical review of the literature on the coloring matter in egg yolk. Poultry Sci. 11:83-93. 1932.

33. Mattson, Fred H. and Harry J. Deuel, Jr. Studies on carotenoid metabolism. III. The effect of a high vitamin A diet on carotenoid metabolism of chickens. Jour. Nutrition 25:103-112. 1943.

34. Maw, W. A. and N. Nikolaiczuk. The effect of ethyl alcohol and trioresol on the vitamin A content of the blood and liver of the chicken. Canadian Jour. Res. Sect. D Zool. Sci. 20:47-49. 1942.

35. Palmer, L. S. Xanthophyll, the principle yellow pigment of the egg yolk, body fat, and blood serum of the hen. The physiological relation of the pigment to the xanthophyll of plants. Jour. Biol. Chem. 23:261-279. 1915.

36. Palmer, L. S. Carotenoids and related pigments. New York: American Chemical Society Monograph Series No. 9. 1922.

37. Palmer, L. S. The biological and chemical nomenclature for the carotenoids. Science 79:488-490. 1934.

38. Palmer, L. S. and H. L. Kempster. Relation of plant carotenoids to growth, fecundity and reproduction of fowls. Jour. Biol. Chem. 39:299-312. 1919A.

39. Palmer, L. S., and H. L. Kempster. The physiological relation between fecundity and the natural yellow pigmentation of certain breeds of fowls. Jour. Biol. Chem. 39:313-330. 1919B.

40. Palmer, L. S. and H. L. Kempster. The influence of specific feeds and certain pigments on the color of the egg yolk and body fat of fowls. Jour. Biol. Chem. 39:331-337. 1919C.

41. Peterson, W. J., J. S. Hughes and L. F. Payne. The carotenoid pigments. Kansas Agr. Expt. Sta. Tech. Bul. 46:1-74. 1939.

42. Rice, James E., Goldan O. Hall, and Dean R. Marble. Judging poultry for production. New York: John Wiley and Sons, Inc. 1930. 425 pp.

43. Riddle, Oscar. On the formation, significance and chemistry of the white and yellow yolk of ova. Jour. Morph. 22:455-485. 1911.

44. Riddle, Oscar. Studies on the physiology of reproduction in birds. I. The occurrence and measurement of a sudden change in rate of growth of avian ova. Amer. Jour. Physiol. 41:387-396. 1916.

45. Romanoff, A. L. Growth and chemical composition of ovum of functioning fowl's ovary (Gallus Domesticus). Biochem. Jour. 25:994-996. 1931.

46. Rubin, Max and H. R. Bird. Some experiments on the physiology of vitamin A storage in the chick. Poultry Sci. 20:291-297. 1941.

47. Rubin, Max and H. R. Bird. The apparent antagonism between vitamin A and carotenoids in the fowl. Science 103:584-586. 1946.

48. Rubin, Max, H. R. Bird and H. M. DeVolti. Avitaminosis A in commercial poultry flock. Poultry Sci. 20:155-160. 1941.

49. Russell, Walter C., M. Wight Taylor, Harry A. Walker and Louis J. Polskin. The absorption and retention of carotene and vitamin A by hens on normal and low fat rations. Jour. Nutrition 24:199-211. 1942.

50. Russell, Walter C. and Albert L. Weber. Plant pigments in the nutrition of the chicken. Proc. Soc. Exp. Biol. and Med. 29:297-298. 1931.

51. Schumacher, A. E., H. M. Scott, J. S. Hughes, and W. J. Peterson. The role of carotenols in growth and reproduction in the fowl. Poultry Sci. 23:529-532. 1944.

52. Schunck, C. A. The xanthophyll group of yellow coloring matters. Proc. Royal Society 72:165-176. 1903.

53. Sherwood, R. M. and G. S. Fraps. Carotene destroying power of certain animal feeds. Poultry Sci. 20:472. 1941. An abstract.

54. Sjollema, B. and W. F. Donath. The vitamin A, carotene and xanthophyll content of the yolk of hens eggs. Biochem. Jour. 34:736-746. 1940.

55. Snedecor, George W. Statistical Methods. Ames: The Iowa State College Press, 1946. 485 pp.

56. Strain, Harold H. Leaf xanthophylls. Carnegie Institute of Washington Publication 490. 1938. 147 pp.

57. Taylor, M. Wight and Walter C. Russell. The provitamin A requirement of growing chickens. Poultry Sci. 26:234-242. 1947.

58. Thudicum, J. L. W. Results of researches on luteine and the spectra of yellow organic substances contained in animals and plants. Proc. Royal Society, London 17:253-256. 1869.

59. Wald, George and Hyman Zussman. Carotenoids of the chicken retina. Jour. Biol. Chem. 122:449-460.

60. Warren, D. C. and R. M. Conrad. Growth of the hen's ovum. Jour. Agric. Res. 58:875-893. 1939.

61. Willstatter, Richard and Heinr, H. Escher. Lutein obtained from egg yolks. Zeit. f. Physiol. Chem. 76:214-225. 1912.